The Journal of LABORATORY and CLINICAL MEDICINE

VOL. 54 AUGUST, 1959 NO. 2

The Official Publication of the Central Society for Clinical Research

Clinical and Experimental

A Method for Increasing the Sensitivity of the Rose Bengal I^{131} Liver Function Test With the Use of Bromsulphalein. Ismael Mena, M.D., Raymond Kivel, M.D., Paul Mahoney, M.D., Sherman M. Mellinkoff, M.D., and Leslie R. Bennett, M.D., Los Angeles, Calif. ---------- 167

A Clinically Useful Liver Function Test Using Radioactive Rose Bengal. Joseph L. Westover, M.D., Moses A. Greenfield, Ph.D., and Amos Norman, Ph.D., Los Angeles, Calif. ---------- 174

The Erythrocyte Uptake of I^{131}-Labeled l-Triiodothyronine as a Measure of Thyroid Function. Alvin L. Ureles, M.D., and Martin Murray, M.S., Rochester, N. Y. 178

A Note Concerning Sources of Variance in Determinations of Human Plasma Volume. John E. Overall, Ph.D., and Clyde M. Williams, M.D., Ph.D., Chapel Hill, N. C., and Pittsburgh, Pa. ---------- 186

The Value of Simultaneous Urine Bilirubin and Serum Alkaline Phosphatase Analyses in the Diagnosis of Intrahepatic Metastases of Carcinoma. Edward J. Fitzsimons, Ph.D., Herbert M. Sommers, M.D., and Thomas C. Laipply, M.D., Chicago, Ill. 192

A Study of Lipids in the Cerebrospinal Fluid. IV. The Determination of Free and Total Cholesterol. Wallace W. Tourtellotte, Ph.D., M.D., Beverley A. Skrentny, B.S., and Russell N. DeJong, M.D., Ann Arbor, Mich. ---------- 197

Mucoproteins of Cerebrospinal Fluid and Blood in Neurologic Disorders. A. Zlotnick, M.Sc., M.D., E. Weisenberg, Ph.D., and I. Chowers, M.D., Jerusalem, Israel ---- 207

Diurnal Variability of Serum Cholesterol at Normal and Reduced Levels. William Shapiro, M.D., E. Harvey Estes, Jr., M.D., and Helen L. Hilderman, A.B., Durham, N. C. ---------- 213

Alterations in Respiratory Function During Natural Sleep. Richard I. Birchfield, M.D., Herbert O. Sieker, M.D., and Albert Heyman, M.D., Durham, N. C. ---------- 216

Excretion of Acid Mucopolysaccharides in the Urine of Patients With Malignant Neoplastic Diseases. Clayton Rich, M.D., and W. P. Laird Myers, M.D., New York, N. Y. ---------- 223

Free Amino Acids in Leukocytes From Normal and Leukemic Subjects. G. Y. N. Iyer, M.Sc., Trivandrum, India ---------- 229

The Hemolytic Effect of Primaquine. X. Aldolase and Glyceraldehyde-3-Phosphate Dehydrogenase Activity in Primaquine-Sensitive Erythrocytes. Stanley L. Schrier, M.D., Robert W. Kellermeyer, M.D., Paul E. Carson, M.D., Charles E. Ickes, M.T., and Alf S. Alving, M.D., Chicago, Ill. ---------- 232

(Contents continued on inside front cover)

PUBLISHED BY THE C. V. MOSBY COMPANY, ST. LOUIS 3, MISSOURI

Contents—*(Continued from front cover)*

Antigenicity of Human Vascular Endothelium: Lack of Relationship to the Pathogenesis of Vasculitis. Sergio Piomelli, M.D., Mario Stefanini, M.D., and Rose H. Mele, B.S., Boston, Mass. ---------- 241

Serial Determinations of Serum Glycoproteins and Proteins in the Early Stages of Experimental Tuberculosis. Hisako Nishihara, D.M.Sc., Seymour Froman, Ph.D., and Henry E. Weimer, Ph.D., Los Angeles, Calif. ---------- 257

The Relationship of the Cation and Water Content of Red Blood Cells to Plasma Composition. H. G. Keitel, M.D., and H. B. S. Jones, B.S., Bethesda, Md. ---------- 262

Urinary Specific Gravity Versus Total Solute Concentration: A Critical Comparison. I. Studies in Normal Adults. Edgar J. Schoen, M.D., George Young, A.B., and Arthur Weissman, J.D., Oakland, Calif. ---------- 277

Laboratory Methods

The Use of the Fluorescent Antibody Technique for the Demonstration of Erythrocyte Antigens. JoAnne Whitaker, M.D., Wolf W. Zuelzer, M.D., Abner R. Robinson, M.S., and Margaret Evans, M.S., Detroit, Mich. ---------- 282

Measurement of Proteolytic Activity of Blood With a Substrate Tagged With Radioactive Iodine. J. C. Heuson, M.D., Brussels, Belgium ---------- 284

Rapid Electrophoretic Separation of Hemoglobins. B. Zak, Ph.D., E. M. Eggers, M.D., T. L. Jarkowski, M.D., and L. A. Williams, B.S., Detroit, Mich. ---------- 288

Photoelectric Ultraviolet Photometry Applied to Protein Electrophoresis. Newton Ressler, Ph.D., Eloise, Mich. ---------- 291

A Rapid Method for Preparing Washed Red Cells Tagged With Chromium^{51}. Chalom A. Albert, M.D., Harold N. Eccleston, Jr., M.D., Amir Rafii, M.D., Charles H. Hunter, M.D., Evelyn E. Henley, M.D., and Solomon N. Albert, M.D., Washington, D. C. ---------- 300

Use of an Artificial Standard for Hemoglobin Determination. Newton Ressler, Ph.D., Norman A. Nelson, M.D., and Irene M. Smith, B.S., Eloise, Mich. ---------- 304

The Estimation of Total Body Water Using a Colorimetric Method for the Determination of Antipyrine (1, 5-Dimethyl-2-Phenyl-3-Pyrazolone) in Plasma. D. Mendelsohn, M.B.B.Ch., B.Sc. (Hons.), and N. W. Levin, M.B.B.Ch., Johannesburg, South Africa ---------- 311

Identification of Barbiturates by Paper Chromatography. Russell Hilf, Ph.D., George A. Lightbourn, M.S., and Frank F. Castano, Ph.D., Fort Baker, Calif. ---------- 320

A Simplified and Exact Method for the Controlled Substitution of Proteins With I^{131}. Norman D. Lee, Ph.D., and Ralph Wiseman, Jr., M.S., Memphis, Tenn. With the Technical Assistance of Ada M. Oldham, B.S. ---------- 325

A Mold for Production of Agar Diffusion Plates. R. L. Miller, B.S., and Robert J. Heckly, Ph.D., Berkeley, Calif. ---------- 333

(Editorial and Business Communications on page 34)

Editor

WILLIAM D. ROBINSON, M.D.

Associate Editor

WILLIAM H. BEIERWALTES, M.D.

University Hospital
University of Michigan Medical School
1313 Ann Street, Ann Arbor, Michigan

Board of Editors

FRANK H. BETHELL, M.D.
University of Michigan, Ann Arbor

WILLIAM H. DAUGHADAY, M.D.
Washington University, St. Louis

ELMER L. DeGOWIN, M.D.
State University of Iowa, Iowa City

RICHARD V. EBERT, M.D.
University of Arkansas, Little Rock

ROBERT H. FURMAN, M.D.
University of Oklahoma, Oklahoma City

ROBERT A. GOOD, M.D.
University of Minnesota, Minneapolis

ROBERT M. KARK, M.D.
University of Illinois, Chicago

RICHARD L. LANDAU, M.D.
University of Chicago, Chicago

GORDON MEIKLEJOHN, M.D.
University of Colorado, Denver

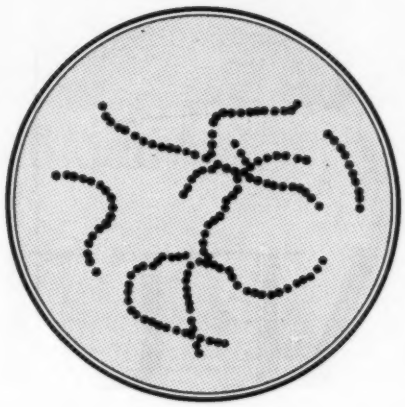
OSCAR D. RATNOFF, M.D.
University Hospitals, Cleveland

DONALD W. SELDIN, M.D.
University of Texas, Dallas

WILLIAM A. SODEMAN, M.D.
Jefferson Medical College, Philadelphia

Vol. 54, No. 2, August, 1959. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo. Subscription rates: United States and its Possessions $13.00, Students $6.50; Canada, Latin-America, and Spain $13.50, Students $7.00; Other Countries $14.50, Students $8.00. Single copies $2.00 postpaid. Entered as Second Class Matter October 8, 1915, at the Post Office at St. Louis, Missouri, under Act of March 3, 1879. Printed in the U. S. A. Copyright © 1959 by The C. V. Mosby Company.

DIFCO



A
S
T
O

Difco reagents for the diagnosis of

Group A Streptococcal Infections

- RHEUMATIC FEVER
- GLOMERULONEPHRITIS

ANTISTREPTOLYSIN *O* TITERS (ASTO) and their relation to pathological conditions in Group A streptococcal infections have established the importance of this determination as a routine clinical test.

Bacto-Streptolysin O Reagent- a dehydrated, standardized and stable reagent requiring only rehydration with distilled water. Antistreptolysin *O* titers have been impractical for routine diagnosis because of the difficulties in preparing the reagent. Bacto-Streptolysin *O* Reagent is a standardized preparation permitting the routine performance of this diagnostic test in all clinical laboratories.

Bacto-ASTO Standard- an antiserum titred in Todd units for use as a control in the determination of antistreptolysin *O* titers.

Descriptive literature sent upon request

DIFCO LABORATORIES

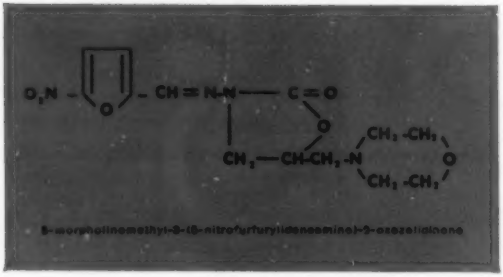
DETROIT 1, MICHIGAN

Announcing

ALTAFUR™

brand of furaltadone

a new member in the nitrofuran family



The first nitrofuran effective orally in systemic bacterial infections

The promise of ALTAFUR *in clinical medicine*

Extensive laboratory and clinical investigative effort has been devoted to the screening and evaluation of nitrofuran compounds in the quest for agents with systemic antibacterial effectiveness. ALTAFUR, a most interesting and promising candidate, resulted from this program.

The in vitro spectrum of ALTAFUR encompasses a variety of pathogenic gram-positive and gram-negative organisms:

Organism (isolated from clinical infections)	*Sensitive*	*Resistant*	*% Sensitive*
Staphylococci*	181	1	99.4
Streptococci	65	1	98.5
D. pneumoniae	14	0	100.0
Coliforms	34	3	91.8
Proteus	5	5	50.0
A. aerogenes	8	0	100.0
Ps. aeruginosa	5	4	55.5

*Includes many strains resistant to antibiotics.

As with other nitrofurans development of bacterial resistance is negligible.

Clinically, ALTAFUR has proven most effective in treating a variety of conditions including pulmonary infections (such as pneumonia, empyema, bronchiolitis), upper respiratory infections, abscesses, cellulitis, pyodermas, septicemia/bacteremia and various wound infections. ALTAFUR has produced cures in 75% of cases, and significant improvement in an additional 10%. It has been used most extensively in staphylococcal infections with a cure rate of 66% and an improvement rate of 20%. Of particular importance, a number of patients had not responded to therapy with antibiotics or other chemotherapeutic agents.

In common with the other available nitrofurans, ALTAFUR has a low order of side effects. Nausea and emesis occur occasionally; these can be minimized or eliminated through dosage adjustment and by giving the drug with meals and with food or milk on retiring. In the two instances in which a neutropenia developed, ALTAFUR was not clearly implicated. There has been no cross-sensitization with other antibacterials.

The average adult dose is one 250 mg. tablet q.i.d. with meals and food or milk at bedtime. For severe staphylococcal infections, the dosage may be increased to approximately 30 mg./Kg. (13.5 mg./lb.) body weight per day, administered in four equally divided doses. The average length of therapy is 5-7 days. Longer therapy may be indicated in the more severe and more complicated infections, such as meningitis, osteomyelitis, bacteremia/septicemia and endocarditis. Additional information may be obtained from the Medical Director, Eaton Laboratories.

ALTAFUR is available as quadrisected, chartreuse-colored tablets of 50 and 250 mg. ALTAFUR Sensi-Discs, for bacterial sensitivity tests, are available from Baltimore Biological Laboratory.

NITROFURANS—a *unique* class of antimicrobials—neither antibiotics nor sulfonamides

EATON LABORATORIES, NORWICH, NEW YORK

NEW

Tudor Modular Cabinets

BY

LAB-TEK

Modules "stack-file" in one compact unit.

Safe, Accurate and Convenient

Tudor Modular cabinets — precision engineered for laboratory filing and storage. Durably constructed of heavy gauge steel with reinforced corners, double-wall panels and hi-impact plastic drawers. Stacked in a vertical column, they use less than 2 sq. ft. of floor space. Doors have hideway design which permits complete access to plastic filing drawers. Smooth rolling castered base permits easy cabinet movement.

TISSUE-TEK 1000

Simplified, compact storing and filing for Tissue-Tek embedding rings. Deluxe metal cabinet has 6 hi-impact plastic drawers to provide storage for 1000 rings.

MICROSLIDE 2000

2000 standard 3" x 1" slides stored in polyurethane-lined compartments. Drawer liners cushion slides and assist convenient "pop-out" removal. Vented design allows complete drying of cover slip mounting media.

2"x2" PHOTOSLIDE 3000

Stores 3000 standard 2"x2" photoslides. Six plastic drawers in each Tudor Module.

For safe filing and compact storage, use versatile Tudor Modular Cabinets by Lab-Tek!

LAB PLASTICS CO. TEK

Contact your Lab-Tek Dealer or write

LAB-TEK PLASTICS CO.

39 East Burlington Ave.
Westmont, Ill.

HYLAND HAEMOPHILUS INFLUENZAE TYPING SERUMS

Types a, b, c, d, e, f

Supplied in 1 cc dropper vials at $10.00 per vial

These serums, *marketed only by Hyland Laboratories*, have been processed to insure potency and type specificity for the capsular swelling test (Neufeld "quellung" reaction). They are used to identify *H. influenzae* in cultures isolated from spinal fluid and other specimens, and have special usefulness in pediatric and contagious disease hospitals.

HYLAND DIAGNOSTIC FEBRILE ANTIGENS

For slide or tube test

Typhoid H Antigen
Typhoid O Antigen
Paratyphoid A Antigen
Paratyphoid B Antigen
Brucella Antigen
Proteus OX-2 Antigen
Proteus OX-19 Antigen
Proteus OX-K Antigen

For tube test only

Tularemia Antigen

FOR RAPID SLIDE (SCREENING) TEST

Supplied in 5 cc vials, complete with dropper which delivers 30 drops per cc, at $3.00 per vial. Glass slide (30 ceramic rings) available $1.25 per slide.

FOR TUBE TEST TITRATION

Supplied in 5 cc vials in concentrated form, equivalent to 50 cc diluted antigen ready for use. $5.00 per vial—except for Tularemia Antigen, which is $6.00 per vial.

HYLAND

Hyland

HYLAND LABORATORIES

4501 Colorado Boulevard, Los Angeles 39, California
160 Lockwood Avenue, Yonkers, New York

BENCH-TOP

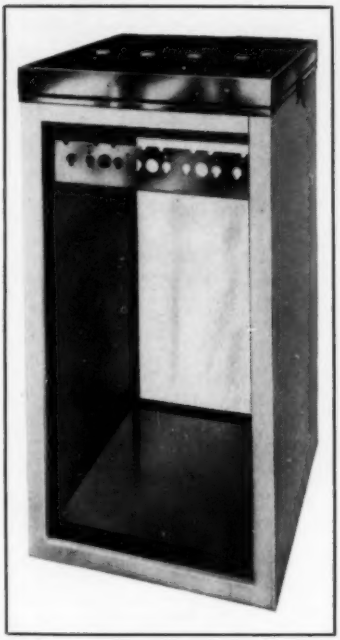
"CHROMATOCAB"

FOR PAPER CHROMATOGRAPHY

Replace your breakable jars with a permanent cabinet of like size!

This Bench-Top Chromatocab accommodates four solvent assemblies of the same size as used in 12 in. square glass jars and in addition has the following advantages:

1. **BETTER INSULATION—**
   Glass wool and double-pane windows!
2. **POSITIVE VAPOR SEAL—**
   Lid sealed with neoprene gasket and clamps!
3. **NO RACK—**
   Suspension of solvent assemblies!
4. **ALL-GLASS SOLVENT ASSEMBLIES—**
   Interchangeable in 12″ square jars!
5. **EASY PORTABILITY—**
   Compact size, light weight!
6. **LESS RISK OF BREAKAGE—**
   than with expensive jars!



CASING—outside casing is sheet steel with white baked enamel finish. It covers a frame which supports the windows and leaves insulation space behind the casing. Rests on 4 rubber feet to prevent scratching of bench.

LID—The lid is faced on both sides with black formica, and the edge is trimmed with stainless steel. It rests on a neoprene gasket fastened to the top of the cabinet, and effects a positive vapor seal by being held down tightly with 2 metal clasps on opposite sides of the casing. Four formica lined holes with snap-caps of polyethylene provide for introduction of solvent through the closed lid.

WINDOW—A full window occupies each of two opposite sides of the cabinet. These are double-paned and sealed in neoprene gaskets.

INSULATION—An inch of glass wool between the outer casing and the inner lining, a ¾ in. dead air space between panes, and an inch thickness of plywood in the lid provide insulation superior to that of glass jars.

SOLVENT ASSEMBLIES—Type O, all glass solvent assemblies are provided, since the style of shelf bracket used permits the trough to be supported on a pair of glass rods instead of in a metal cradle.

DIMENSIONS—Outside: 14″ x 15″ x 26″ high—inside: 11½″ x 12″ x 24″. Deep—Window: 12″ x 24″ x ⅛″ thick, ¾″ space between panes.

Cat. No. M-46414 BENCH-TOP CHROMATOCAB — complete with four solvent assemblies.

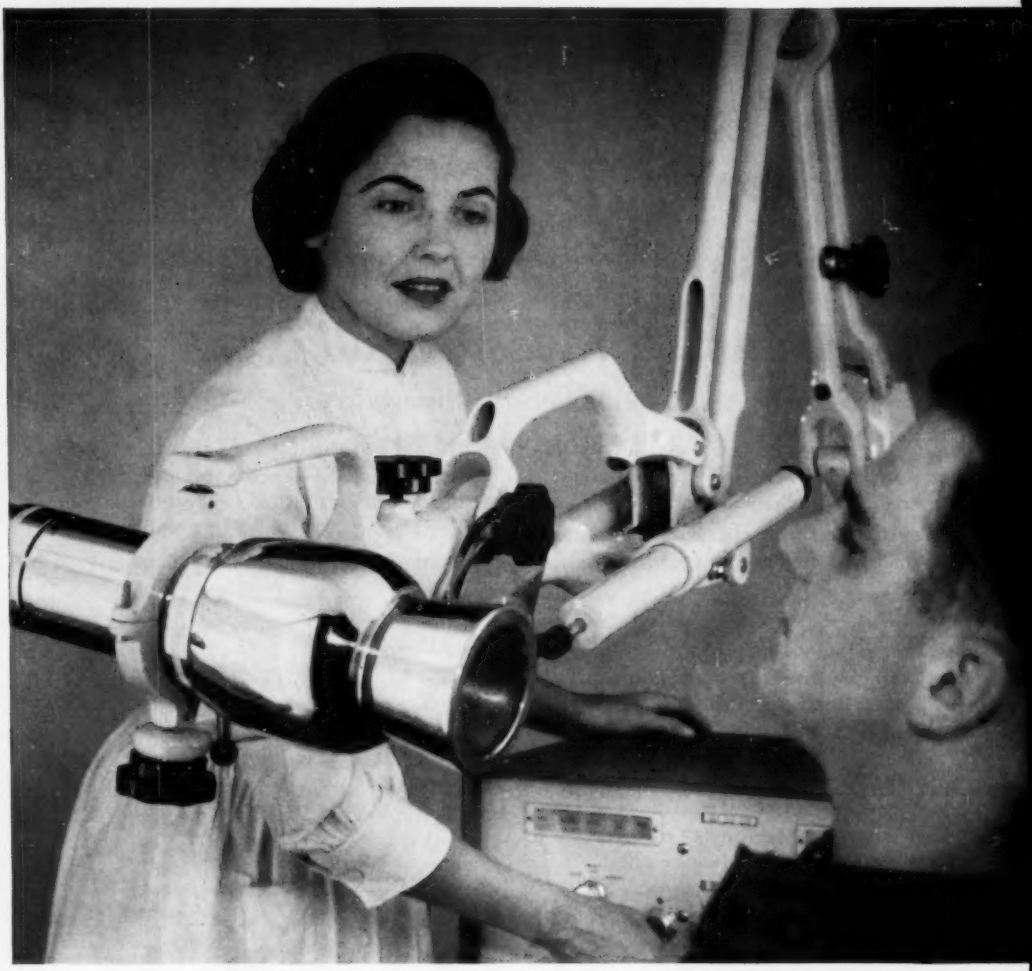
Each $115.00

STANDARD SCIENTIFIC SUPPLY CORP. • 1888 •

STANDARD SCIENTIFIC Supply Corp.

808 BROADWAY
NEW YORK 3, N.Y.

LABORATORY APPARATUS
REAGENTS AND CHEMICALS



Nuclear-Chicago's experience in manufacturing medical instruments for detecting and measuring radioactivity began with the first hospitals to employ radioisotopes in diagnostic tests. From that time on, *quality* and *dependability* in design, manufacture, and service have made us the world's leading supplier of instruments for nuclear clinical and diagnostic use in medicine. Our specially trained medical sales and service force covers every state and most countries of the free world.

We invite you to call on our experience at an time for assistance in initiating radioisotop procedures or in modernizing your presen facilities to cover new applications of radi isotopes. Our General Catalog R describes i detail over fifty medical instruments, acce sories, and complete laboratories to cover ever possible need in diagnostic applications radioisotopes. If you would like to receive personal copy for your files, just drop us card or letter—there is no obligation.

Fine Instruments - Research Quality Radiochemicals

nuclear - chicago
CORPORATION
249 WEST ERIE STREET • CHICAGO 10, ILLINOIS

"TO KNOW IS FIRST TO MEASURE"

Sodium
Potassium } *The Coleman Flame Photometer*
Calcium *Measures all 3*

The Coleman Flame Photometer today stands unchallenged as the most effective and most widely used instrument for flame analysis in the clinical and biological laboratories of the world. Its precision, simplicity and absolute dependability have been established beyond question. These are some of the characteristics that have gained for it the position of high regard that it occupies among Pathologists, Medical Technologists and Clinical Chemists alike:

Measures all 3
Calcium, as well as sodium and potassium, without requiring separation or chemical pre-treatment other than simple dilution of the liquid sample.

Direct Reading
Sample concentrations are read directly in milliequivalents per liter of sample. No calibration curves, tables or calculations are necessary.

Minimum Sample Volumes
Replicate measurements of serum Na and K need only 0.02 ml of serum; 0.1 ml suffices for Ca analysis.

Fast Readings
Stable and reproducible readings in less than 15 seconds.
No tedious waiting for meter equilibrium.

Note:
With the Coleman Flame Photometer the use of Lithium, or other "internal standard" additions to sample solutions is unnecessary.

For practical and dependable flame analysis of biological materials:
The Coleman Model 21 Flame Photometer. Ask for Bulletin B-242

COLEMAN INSTRUMENTS, INC., DEPT. A, MAYWOOD, ILL.

Paragon Stains Paramount Quality

Tested and proven stains of the very highest quality

Paragon special stains are stable, fresh and ready instantly when special staining is required

OIL RED O FAT STAIN—PARAGON

For brilliant red fat stains on frozen sections, fecal smears, etc., of startling intensity unsurpassed by any other method. There is no loss of fat during staining. The technic is rapid and not critical. Polyethylene Glycol is required in the staining technic.

PS1266	*Oil Red O Fat Stain—Paragon*	*Bottle (100 cc)*	*$2.15*
PS1268	*Polyethylene Glycol—Paragon, U.S.P.*	*Bottle (250 cc)*	*1.20*

MUCICARMINE STAIN COMPOUND—PARAGON

is a powdered stain instantly soluble in 50% alcohol to prepare Mayer's Mucicarmine stain for mucin. No Heating. No Filtering. Stable. Each gram makes 50 cc of staining solution.

PS1501	*Mucicarmine Stain Compound—Paragon*	*Bottle (10 grams)*	*1.75*

BEST'S CARMINE STAIN COMPOUND—PARAGON

is a dry powder requiring only the addition of ammonia and methyl alcohol to prepare the working solution for the staining of glycogen in paraffin sections and cytological smears. Dissolves readily. No Heating. No Filtering. No Ageing. Ready at once.

PS1505	*Best's Carmine Stain Compound—Paragon*	*Bottle (25 grams)*	*2.75*

GIEMSA STAIN—PARAGON (Stock Stain Solution)

Carefully prepared, stable, ready to use, economical.
One cc makes 50 cc working stain. Ideal for blood and bone marrow as well as malarial and blood parasites.

PS1160	*Giemsa Stain—Paragon*	*Bottle (25 cc)*	*1.00*
PS1161	*Giemsa Stain—Paragon*	*Bottle (100 cc)*	*2.75*

PARAGON BUFFER SOLUTION FOR GIEMSA STAIN

Ready to use buffer solutions which adjust the Giemsa working stain to the optimum pH for proper staining. For blood and bone marrow use pH 6.5; for malaria and protozoa use pH 6.8.

PS1165	*Paragon Buffer Solution For Giemsa Stain pH 6.5*	*Bottle (100 cc)*	*1.00*
PS1166	*Paragon Buffer Solution For Giemsa Stain pH 6.5*	*Bottle (250 cc)*	*1.50*
PS1168	*Paragon Buffer Solution For Giemsa Stain pH 6.8*	*Bottle (100 cc)*	*1.00*
PS1169	*Paragon Buffer Solution For Giemsa Stain pH 6.8*	*Bottle (250 cc)*	*1.50*

Write for catalogs 957A & 957B for more detailed information and staining technics

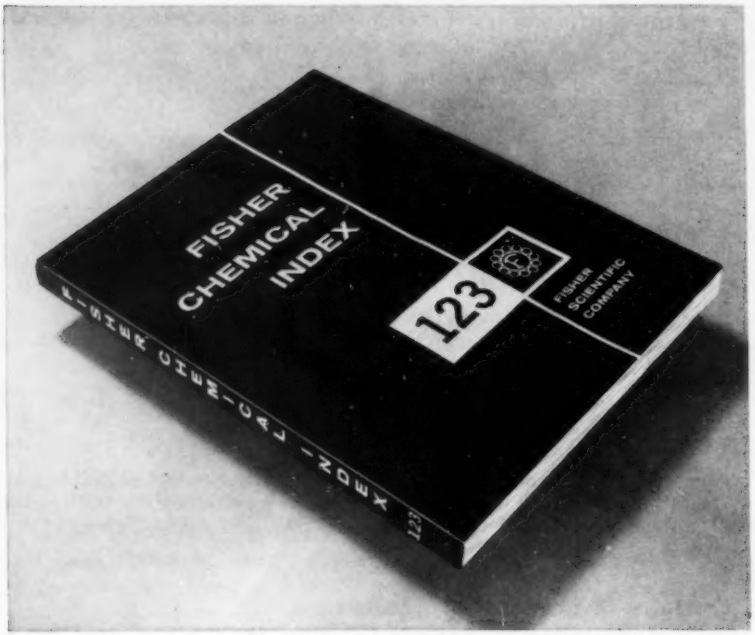
All prices F. O. B. New York, New York, subject to change without notice

Manufactured exclusively by

PARAGON C. & C. CO. INC.

2540 Belmont Avenue, New York 58, New York

Cable address: Wijeno, New York



New! 1959 Edition

FISHER CHEMICAL INDEX

All your chemical needs from one source. Over 7,000 products normally used in the laboratory are listed in the new *Fisher Chemical Index.*

The new *Index* lists almost every known chemical that is available commercially and of use to science, in a simple A-to-Z listing. For the convenience of bacteriologists and others in the health sciences, the *Index* includes a comprehensive alphabetical listing of culture media and allied reagents.

The *Index* describes hundreds of Fisher "Certified" Reagents, chemicals produced and individually lot-analyzed for guaranteed high purity in the Fisher Chemical Manufacturing Division, America's largest reagent plant. (*All* of the ACS specified reagents are included in the Certified line.)

If you haven't received your copy of the 1959 *Fisher Chemical Index*, write today.

B-100b

120 Fisher Building, Pittsburgh 19, Pa.

FISHER SCIENTIFIC

IN THE U.S.A.
Boston
Buffalo
Charleston, W.Va.
Chicago
Cleveland
Detroit
New York
Philadelphia
Pittsburgh
St. Louis
Washington
IN MEXICO
Mexico City
IN CANADA
Edmonton
Montreal
Toronto

America's Largest Manufacturer-Distributor of Laboratory Appliances & Reagent Chemicals

Whatever you're filtering, fritted ware helps you filter it better

Whatever you're filtering—coarse materials, crystalline or fine precipitates, floaters, or bacteria—a PYREX fritted filter will help you do it faster, more efficiently.

There is a wide variety of laboratory glassware into which have been sealed porous-glass filters. Filters are available in six porosities ranging from 0.9 to 220 microns. Each porosity is closely controlled to retain only those particles that are greater than stated pore size.

You can keep PYREX fritted ware ready to use at a moment's notice. No need to fold, fit and trim papers. Just hook up the vacuum and begin.

You'll use your fritted glassware indefinitely. It's strong. It's easily cleaned with chemicals or by backwashing. PYREX fritted ware is made of our chemical glass No. 7740; it resists corrosive reagents, takes repeated sterilizations, cannot contaminate delicate solutions.

You'll find the initial cost of PYREX fritted ware quite reasonable. Consider the re-use and convenience factors and you'll say it's downright economical. And, for even greater savings, you can include it with your regular PYREX labware order.

For details on the complete line, contact your laboratory supply representative or consult PYREX labware catalog LG-1. If you don't have a copy, please send the coupon.

In use, item 33990, Morton Bacteria Apparatus with fritted disc. In background, tubular filter for laboratory or pilot plant production. Other items of interest in the catalog include: 36060. Buchner Funnel with fritted disc; 38450 and 38460, Chromatographic Tubes with coarse-porosity fritted disc.

CORNING GLASS WORKS
82 Crystal Street, Corning, N. Y.

Please send ☐ LG-1 PYREX Laboratory Ware
☐ Supplement No. 3 to Catalog LG-1

Name..... Title.....
Company.....
Address.....
City..... Zone..... State.....

PYREX® laboratory ware ... *the tested tool of modern research*

For Better Microtome Knife Maintenance

Aloe Precision Electric Sharpener and Micro-Stropper

- ***Sharpener produces perfect edges in less than 5 minutes.***
- ***Micro-Stropper reduces sharpening and grinding requirements—increases knife life.***

Three stick abrasives are furnished with sharpener for specific type knife edges. No mess involved as with water abrasives. Sharpener provides keener, longer lasting cutting edges and maintains original knife bevel. Stropper assures perfectly straight, uniform

edge every time. Together, sharpener and stropper offer the ideal knife maintenance assembly—at a price below that of most sharpeners alone.

Complete information sent on request. Write or call us today.

aloe

OUR 100th YEAR

aloe scientific

DIVISION OF THE BRUNSWICK-BALKE-COLLENDER COMPANY

General Offices: 5655 Kingsbury, St. Louis 12, Mo.

Fully stocked divisions coast to coast

add confidence

to your

cross match

ANTI-HUMAN SERUM

FOR THE COOMBS ANTI-GLOBULIN TEST

Ortho Pharmaceutical Corporation

RARITAN, NEW JERSEY

Ortho

RECORD MANY VARIABLES with the SARGENT RECORDER

(PATENTS PENDING)

Here in one expertly designed instrument is a Multi-purpose Recorder that measures current and voltage and all other quantities which can be transposed into potential or current signals.

THIS RECORDER FEATURES:

18 Current Ranges—18 Potential Ranges
9 Chart Speeds (Time Range)
(27 Chart Speeds with Multi-range Attachment)
Designed for Bench Operation

S-72150 SARGENT RECORDER-$1725.00

YOU CAN RECORD:

Thermal Conductivity	Dielectric Constant
Temperature	Potential
Current	Conductance
	Light Intensity

For complete information write for Bulletin R.

Designed and Manufactured by **E. H. SARGENT & CO.**

SARGENT

SCIENTIFIC LABORATORY INSTRUMENTS • APPARATUS • SUPPLIES • CHEMICALS

E. H. SARGENT & COMPANY, 4647 W. FOSTER, CHICAGO 30, ILLINOIS
DETROIT 4, MICH. • DALLAS 35, TEXAS • BIRMINGHAM 4, ALA. • SPRINGFIELD, N. J.

Paragon Stains — Paramount Quality

Tested and proven stains of the very highest quality

PAPANICOLAOU STAINS—PARAGON

EA-36 EA-65 OG-6
Harris Hematoxylin (modified)

Papanicolaou Stains prepared according to the original formulae for the cytological diagnosis of cancer by means of the smear technic.

These stains conform to Paragon's rigid standard of excellence in every way at a modest cost that renders preparation by the laboratory technician unnecessary.

STABLE **READY TO USE**

Each lot of stain is tested against smears in our laboratories for correct differential staining, color balance and transparency.

* * * * * * * * * * * *

PAPANICOLAOU STAIN—PARAGON EA-36
For general staining of vaginal and cervical smears and in endocrine studies.

PAPANICOLAOU STAIN—PARAGON EA-65
For staining smears containing much mucus as sputum, gastric and pleural fluids, etc. Similar to EA-36 but yielding better differentiation in the presence of mucus.

PAPANICOLAOU STAIN—PARAGON OG-6
The Orange G stain for use with EA-36 and EA-65 in the Papanicolaou technic.

HARRIS HEMATOXYLIN—PARAGON (modified)
For Papanicolaou Staining
A modified ready to use Harris Hematoxylin Stain specially formulated for Papanicolaou staining. It yields a sharp blue nuclear stain with no staining of the cytoplasm.

* * * * * * * * * * * *

PAPANICOLAOU STAINS—PARAGON are packed in two convenient sizes only, a 250 c.c. and a 500 c.c. bottle.

Name	Catalog No.	500 c.c. Bottle	250 c.c. Bottle
HARRIS HEMATOXYLIN—PARAGON (modified)	PS1281	$2.25	
For Papanicolaou Staining	PS1291		$1.50
PAPANICOLAOU STAIN—PARAGON EA-36	PS1282	3.85	
	PS1292		2.35
PAPANICOLAOU STAIN—PARAGON EA-65	PS1283	3.85	
	PS1293		2.35
PAPANICOLAOU STAIN—PARAGON OG-6	PS1284	3.25	
	PS1294		2.00

Write for catalogs 957A & 957B fully describing Paragon Staining Solutions, Chemical Reagents and Solutions and Paragon Tray Drawer Cabinets and accessories

* * * * * * * * * * * *

All prices F.O.B. New York, New York, subject to change without notice.

Manufactured exclusively by

PARAGON C. & C. CO., INC., 2540 Belmont Ave., New York 58, N.Y.

Cable address: Wijeno, New York

You remove one slide at a time ...you touch only the edges

- This new carton is the first effective answer to the challenging problem of fast, convenient slide dispensing.
- Single slide exposed each time; remaining slides preserved in original clean condition.
- Each slide is individually cleaned to exceptional standards, permitting immediate use.
- Made of the finest glass. No sharp edges . . . ground smooth for safe handling.
- Adhesive strip on bottom of carton holds dispenser on any flat surface.

S/P Protecta-Slide, 1 x 3 inches, 1.2 mm thick. Packed 72 slides per dispenser.

No. 66521—Plain.....Gross, **$2.25**
25 gross, per gross, **2.00**

No. 66522—Frosted.....Gross, **$3.10**
25 gross, per gross, **2.80**

Ask the man who knows slides best...have your S/P representative show you his samples

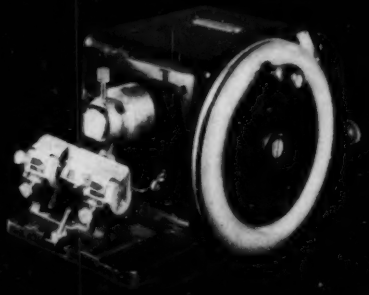
Protecta-Slide—
T.M. S/P, Div. AHSC

Scientific Products

DIVISION OF AMERICAN HOSPITAL SUPPLY CORPORATION

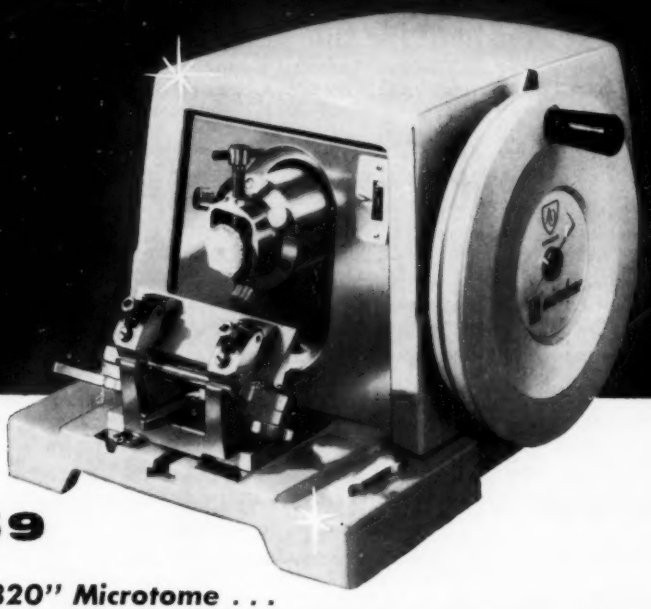
GENERAL OFFICES: 1210 LEON PLACE, EVANSTON, ILLINOIS

Regional Offices: Atlanta • Chicago • Columbus • Dallas • Kansas City • Los Angeles • Minneapolis • New York • San Francisco • Washington



1903

This is the first Spencer "820" Microtome ever made (Serial No. 1). Sold in 1903 and traded in on a new "820", it still cuts consistently uniform sections . . . after over half a century of use.



1959

Today's "820" Microtome . . .
Improved performance plus new convenience

The new "820" has a striking new look . . . and important new convenience features.

SETTING—The feed indicator is positioned at the front of the instrument for easier checking of settings.

LOCKING—A new top-positioned locking lever means quicker and safer locking of the drive wheel.

CLEANING—New cast-aluminum cover swings back to expose the entire inner mechanism for easier cleaning of wax and debris.

LUBRICATION—New and readily accessible lubrication points make preventive maintenance easy.

The inner mechanism and operating principle of this new "820" remain unchanged except for one important improvement. New construction and quality control methods make it possible to fit slideways to closer tolerances than ever before! This precision performance assures the uniform serial sections so necessary for successful diagnosis of pathological conditions.

CARRY A SPARE!

How old is your present "820"? Perhaps now is the time to purchase a new, improved "820". Our factory can recondition your old instrument for you to retain as a spare . . . you'll have an extra instrument for use during those frequent rush periods and as insurance against emergencies. This vital "operating" insurance costs only a few cents a day.

See your AO Sales Representative or write for NEW COLOR BROCHURE SB820.

AO SPENCER

American Optical Company

INSTRUMENT DIVISION, BUFFALO 15, NEW YORK

Dept. T15

Please send Brochure SB820 with full information on AO Spencer's "820" Rotary Microtome.

Name_____

Address_____

City_____ Zone____ State_____

CRAIG and FAUST'S

CLINICAL PARASITOLOGY

By ERNEST CARROLL FAUST, A.B., M.A., PH.D.

The William Vincent Professor of Tropical Diseases and Hygiene, Department of Tropical Medicine and Public Health, Tulane University School of Medicine, New Orleans, La., Consultant, U.S. Public Health Service; Member, Expert Panel on Parasitic Diseases, World Health Organization

and PAUL FARR RUSSELL, M.D., M.P.H.

Staff Member, The Rockefeller Foundation; Consultant to the Surgeon General, U.S. Army; Member, Malaria Panel and Formerly Chairman, Expert Committee on Malaria, World Health Organization

With the Editorial Assistance of

DAVID RICHARD LINCICOME, B.S., M.S., PH.D.

Howard University, Washington, D.C., Editor of the Journal, Experimental Parasitology

CHANGING populations, especially migration from endemic areas, and greatly increased world travel, have contributed much toward the rising awareness of parasites and tropical diseases in the United States. Northern and eastern areas are not exempt from this potential infiltration. Encouragement lies in the significant modern concept which has been introduced in this edition.

This is a sound, widely recognized text and reference book on protozoölogy, helminthology and arthropods as agents and vectors of disease-producing organisms. Etiology, pathology, symptomatology, diagnosis, treatment, prevention and control are detailed by two eminent authorities in this field. A special *Technical Appendix* gives the technics and aids which are available for diagnosis and control.

What Medical Journals Say About the 6th Edition

"Highly recommended."—*American Journal of the Medical Sciences.*

"If you can use it, buy it—it is worth it."—GP.

"Exceptionally well illustrated. Highly recommended for everyone interested in any phase of parasitology."—*U. S. Armed Forces Medical Journal.*

"The physician and clinical pathologist will find this book authoritative and necessary in the study of the subject."—*Postgraduate Medicine.*

"A front-running candidate for all who work in the field of human parasitology, or who are studying the subject. The authors are to be commended for an outstanding piece of work."—*Tropical Medicine and Hygiene News.*

6th Edition. 1078 Pages. 346 Illustrations and 7 Plates in Color. 23 Tables $15.00

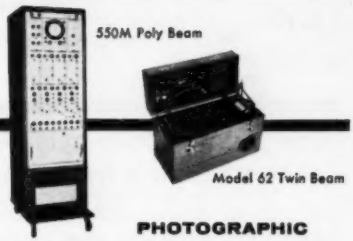
LEA & FEBIGER WASHINGTON SQUARE PHILADELPHIA 6 PA.

FOR ELECTROCARDIOGRAPHY

New Model 100 Viso . . . 2 recording speeds, 3 sensitivities for extreme diagnostic accuracy.

Model 300 Visette . . . 18 lb. portable ECG, "traveling" instrument.

Model 55 EEG/ECG High Gain Preamplifier . . . designed for use with Sanborn 'cardiographs and multichannel recorders for fetal ECG's, EEG (monitoring), small muscle myograms. Fully transistorized, portable unit.



FOR MULTI-CHANNEL RECORDING

DIRECT WRITING

150M series . . . from one-channel benchtop portables to multi-channel systems in mobile cabinets; interchangeable plug-in preamplifiers.

350M series . . . 6 or 8 channels, improved design, frequency response to 120 cps, single cabinet housing, interchangeable plug-in preamplifiers. Optional plug-in monitoring unit (scope, electronic switch, vector timer). Model 60 Twin Viso . . . 2 channel mobile system.

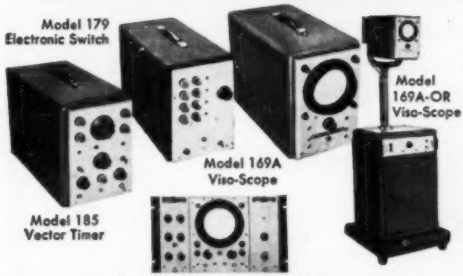
PHOTOGRAPHIC

550M Poly-Beam series . . . 6 or 8 channels with interchangeable plug-in preamplifiers, single cabinet housing, optional plug-in monitoring unit (same as 350M). Model 62 Twin Beam . . . 2-channel system for diagnostic phonocardiograms, ECG'S, etc.

PRESSURE TRANSDUCERS for use with these systems include types for differential or single-ended measurement of liquid or gas pressures; two sensitivities — 1 cm/1mm Hg. or 1 cm/0.1mm Hg.; optional adapters for use with earlier Sanborn systems.

SANBORN

diagnostic/research INSTRUMENTATION



Complete Visual monitoring Unit

FOR VISUAL MONITORING

Model 169A-OR Viso-Scope . . . for ECG monitoring during surgery. Bright, sharp traces; UL approved for use in presence of explosive gases.

Model 169A Viso-Scope . . . portable 'scope primarily for use with the model 179 Electronic Switch, Model 185 Vector Timer.

Model 179 Electronic Switch . . . for viewing up to four traces simultaneously on the Model 169A Viso-Scope.

Model 185 Vector Timer . . . for Vector loop presentation on the 169A Viso-Scope.

Model 569 Viso-Scope, 579 Electronic Switch and 559 Vector Timer in a compact, plug-in form for use with 550M or 350M systems.

FOR HEART SOUND ANALYSIS

Model 278 High Fidelity Tape Recorder/Player . . . for accurate recording and playback of heart sounds.

Model 256 Amplifying Stethoscope . . . high fidelity monitoring of cardiac sounds by groups of up to 13 listeners.

Model 280 Rappaport-Sprague Acoustic Stethoscope . . . new, high efficiency stethoscope with five interchangeable chest pieces for various heart sounds.

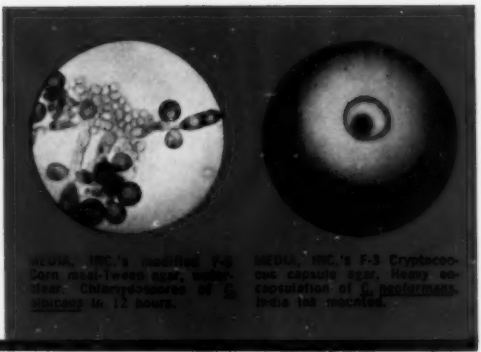
Sanborn Branch Offices and Service Agencies in principal cities throughout the U. S.

For complete information or demonstration of Sanborn instruments, call your local Sanborn representative.

SANBORN COMPANY
MEDICAL DIVISION
175 Wyman Street Waltham 54, Mass.

New from MEDIA, INC.

a full line of highest quality prepared agar media ✻ in disposable plastic tubes and plates ✻ **same size as glass petri dishes** ✻ sterile, ready to use



MEDIA, INC.'s modified F-5 Corn meal-Tween agar, under-clear. Chlamydospores of C. albicans in 12 hours.

MEDIA, INC.'s F-3 Cryptococcus capsule agar. Heavy encapsulation of C. neoformans-India ink mounted.

FOR FUNGAL DIAGNOSIS—exclusive with MEDIA, INC.

F-1 LITTMAN LIVER-SPLEEN GLUCOSE BLOOD AGAR, with penicillin and streptomycin, for rapid primary isolation of pathogenic fungi at 37° C. (Am. J. Clin. Path. 25:1148, 1955)

F-2 UREA AGAR FOR FUNGI, for detection of urea hydrolysis by fungi, especially useful for rapidly differentiating C. albicans from C. neoformans. (J. Inf. Dis. 101:51, 1957)

F-3 CRYPTOCOCCUS CAPSULE AGAR, for ready encapsulation of C. neoformans at 37° C. (Trans. N. Y. Acad. Sci. 20:623, 1958)
Patent applied for.

F-5 CORN MEAL-TWEEN 80 AGAR, for demonstration of chlamydospores

OTHER MEDIA FOR FUNGAL DIAGNOSIS

F-4 LITTMAN OXGALL AGAR
F-6 SABOURAUD DEXTROSE AGAR
F-7 SABOURAUD MALTOSE AGAR
F-8 SABOURAUD DEXTROSE AGAR, with penicillin and streptomycin.

FOR BACTERIAL DIAGNOSIS

B-2 HEART INFUSION BLOOD AGAR
B-5 BRAIN HEART INFUSION GLUCOSE BLOOD AGAR
B-8 MUELLER HINTON AGAR (for gonococci and meningococci)
B-10 MANNITOL SALT AGAR (for staphylococci)
B-11 CHAPMAN STONE AGAR (for staphylococci)
B-12 TRYPTICASE SOY AGAR
B-14 CHOCOLATE BLOOD AGAR
B-15 NUTRIENT AGAR
B-18 SS AGAR (SHIGELLA-SALMONELLA)
B-19 DC AGAR (DESOXYCHOLATE-CITRATE)
B-33 UREA AGAR (for bacteria)

Write today for information on complete line. Any desired formula made to order—specify your needs.

CUT HERE AND USE AS ORDER FORM

CHECK QUANTITY	PLATE	TUBE
F-1		
F-2		
F-3		
F-5		
F-4		
F-6		
F-7		
F-8		
B-2		
B-5		
B-8		
B-10		
B-11		
B-12		
B-14		
B-15		
B-18		
B-19		
B-33		

LIST PRICES (subject to change)
Net 30 days.

No. of a kind	Price per plate	Price per tube
6	$0.60	$0.35
36	0.57	0.32
144	0.54	0.30

MEDIA, INC. originated and developed the new MEDIKIT (tm)—the fast-acting, disposable bacterial sensitivity kit for physicians. Write for illustrated brochure.

MEDIA, INC. 89 LINCOLN PARK NEWARK 2, N. J.

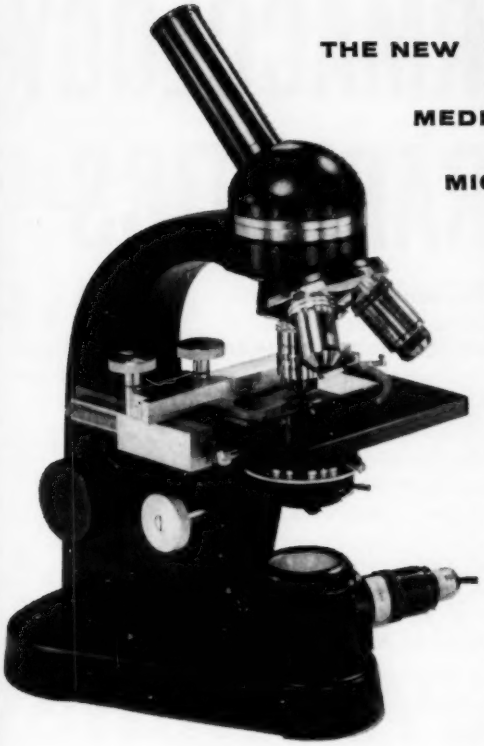
Send to NAME .....

ADDRESS .....

THE NEW Leitz

MEDICAL & LABORATORY

MICROSCOPE **SM**



Leitz sets a new standard in introducing this general purpose microscope for student and laboratory use. The **SM** model embodies the advances of design and craftsmanship that only 100 years of microscope experience can provide. It combines solid construction and operational ease with true accuracy and precision. The Leitz MEDICAL & LABORATORY MICROSCOPE **SM** is the ideal microscope for the laboratory. Among its outstanding features are:

- Famous Leitz ball-bearing, single knob, dual focusing control, combines fine and coarse focusing
- Handsome design, solid and dependable construction
- Flat-surfaced stand of corrosion-resistant light alloy
- May be used turned away from observer for easy accessibility to slide
- Instant-locking device changes tubes (inclined or straight monocular or binocular) in a one-step operation
- Variety of object stages to choose from
- Removable mirror interchanges with attachable illuminators
- Retractable spring-loaded mounts on high-powered objectives provide positive protection against damage to slide or front lens.

A TYPICAL COMBINATION OF ACCESSORIES
MEDICAL & LABORATORY MICROSCOPE **SM**, with inclined monocular tube, mechanical stage; two-lens condenser with swing-out upper element and iris diaphragm; quadruple nosepiece; mirror and fork; carrying case. With optical outfit consisting of achromats 3.5x, 10x, 45x, and 100x—oil 6x and 10x eyepieces **$411.50**

E. Leitz, Inc., Dept. L-8
468 Fourth Avenue, New York 16, N.Y.
Please send me additional information on the **SM** Microscope

NAME_____

STREET_____

CITY_____ ZONE_____ STATE_____

21357

E. LEITZ, INC., 468 FOURTH AVENUE, NEW YORK 16, N.Y.
Distributors of the world-famous products of Ernst Leitz G.m.b.H., Wetzlar, Germany—Ernst Leitz Canada Ltd.
LEICA CAMERAS · LENSES · MICROSCOPES · BINOCULARS

NOW... a **CLINICAL** Journal of Pharmacology

PHARMACOLOGY and THERAPEUTICS

FIRST ISSUE: January 1960

Editor
WALTER MODELL, M.D.

Editorial Board
W. A. Bain, M.D.
Edward A. Carr, Jr., M.D.
Windsor Cutting, M.D.
Arthur C. DeGraff, M.D.
James M. Dille, M.D.
Alan K. Done, M.D.
Dale G. Friend, M.D.
Arthur Grollman, M.D.
Raymond W. Houde, M.D.
Ernest Jawetz, M.D, PhD.
David A. Karnofsky, M.D.
Kenneth G. Kohlstaedt, M.D.
Herbert S. Kupperman, M.D.
D. R. Laurence, M.D., M.R.C.P.
T. A. Loomis, M.D., Ph.D.
Donald Mainland, M.D.
H. Houston Merritt, M.D.
Eric Nilsson, M.D.
Carl C. Pfeiffer, Ph.D., M.D.
Leroy D. Vandam, M.D.
Walton Van Winkle, Jr., M.D.
Gerhard Werner, M.D.

CLINICAL PHARMACOLOGY AND THERAPEUTICS will publish objective and authoritative papers dealing with the evaluation of the effects of drugs in man. It will present comprehensive papers on new drugs and critical re-evaluations of old ones. It will include editorials, symposia, reviews and a correspondence section for informal reports. It will also offer brief commentaries on new pharmaceuticals as they are introduced, with advice and prognostications of authorities, as unbiased guides to their use before extensive clinical experience is available.

Papers to Appear in Early Issues:

The Actions and Uses of Anthelmintics . . . The Respiratory and Circulatory Effects of Opiates and Narcotic Antagonists . . . The Pharmacologic Basis of the Choice and Use of Drugs in Myasthenia Gravis . . . The Present Status of Anabolic Agents . . . The Actions and Uses of Hypoglycemic Agents . . . The Mechanism of Action of Diuretics in Hypertension . . . The Newer Drugs in Amebiasis . . . The Reliability of Reports on Drug Evaluations in Man . . . Experimentation with New Drugs on Man: A Symposium . . . Pharmacology of Muscle Relaxants in Man.

ENROLL NOW WITH THIS COUPON . . . .

Become a charter subscriber to this timely publication devoted exclusively to clinical pharmacology and therapeutics! Take advantage of the generous introductory offer described on the enrollment form.

THE C. V. MOSBY COMPANY, 3207 Washington Boulevard, St. Louis 3, Missouri

Dear Sirs: Date.....

Enroll me as a Charter Subscriber to CLINICAL PHARMACOLOGY AND THERAPEUTICS. Send the first two issues free. Bill me for $12.50 (Canada & Latin America, $13.00; elsewhere, $13.50) for the next six bimonthly issues.

☐ I'm enclosing my check for $..... ☐ Bill me,
(Attach your check; receive 3 issues free!)

☐ I am in General Practice ☐ I specialize in.....

Dr.....

Address.....

City.....Zone.....State.....

Microscopic Mounting and Embedding Media

H·F·M — **HARLECO FLUORESCENCE MOUNTANT**
A ***permanent medium*** for FLUORESCENT MICROSCOPY incorporating a methacrylate base in toluene solution.

Flurol — **HARLECO FLUORESCENCE OIL MOUNTANT**
A mineral oil medium of suitable viscosity for ***temporary*** mounts in FLUORESCENT MICROSCOPY.

H·S·R — **HARLECO SYNTHETIC RESIN**
A superior synthetic resin for permanent specimen mounts. This product has largely replaced the use of time-honored Canada Balsam.

H·I·O — **HARLECO IMMERSION OIL**
A compounded oil with refractive index similar to cedarwood oil. It is water-white and non-drying.

H·E·M — **HARLECO WATER-SOLUBLE EMBEDDING MEDIUM**
A water-soluble "Carbowax" mixture intended for the rapid tissue technic in examination of fresh tissue.

Paraffin Wax — **REFINED FOR EMBEDDING**
Molded in ¼ pound rectangular blocks, four to the package. In 25# shipping cartons for storage. Melting Range 54.5-56.0°C. Also furnished in two grades of lower melting range, medium hard 51.5-54.0°C. and soft 49.0-51.0°C.

All of the above products are of the same fine quality as the many other preparations for the laboratory under the Harleco Brand. General catalog will be sent upon request.

Harleco Products are available of your preferred supply house

HARLECO

HARTMAN-LEDDON COMPANY
60th & Woodland Avenue • Philadelphia 43, Pennsylvania • U.S.A.

ANIMAL CAGES
"econo-cage"

Of moulded, seamless construction, with rounded corners and sloping sides for easy cleaning and convenient nesting for shipping and storage. Fiberglas reinforced plastic is lightweight, resistant to impact and abrasion, withstands repeated autoclaving at 250°F, and is unaffected by commonly used disinfectants and cleaning agents. Its low thermal conductivity avoids chilling of young animals.

Offered in three sizes, each with or without window of methacrylate plastic which can be chemically sterilized but must be removed when autoclaving the cages. Size 12 has reinforced rim, and 22 and 32 have turned rims.

Three types of covers are available, i.e. A of plastic with ventilating holes and either of two sizes of removable round feeder of galvanized wire mesh; B of wire mesh in plastic frame with integral V-shape feeding trough; and flat cover C of wire mesh in plastic frame, without feeder.

Cat. No.	Size	Application	Overall dimensions	Cover	Window	Feeder	Price *Each
1105.	12	General Purpose, for mice, rats, hamsters, guinea pigs, etc.	11 in. long × 8½ in. wide × 6 in. high	A	with	round 23 cu. in.	6.50
1105-B.	12	do.	do.	A	without	do.	6.50
1105-D.	12	do.	do.	B	with	V-shape 53 cu. in.	9.65
1105-F.	12	do.	do.	B	without	do.	9.65
1105-H.	22	Mouse Cage, in accordance with U. S. N.I.H. Spec. FG-84	11½ in. long × 7½ in. wide × 5 in. high	A	with	round 23 cu. in.	6.50
1105-J.	22	do.	do.	A	without	do.	6.50
1105-L.	22	do.	do.	B	with	V-shape 46 cu. in.	9.65
1105-N.	22	do.	do.	B	without	do.	9.65
1105-P.	32	Breeding Cage for mice; also for rats, etc.	19 in. long × 10½ in. wide × 5⅛ in. high	B	with	V-shape 63 cu. in.	13.00
1105-R.	32	do.	do.	B	without	do.	13.00

*Prices on request for lots of 144 or 500 for shipment at one time; also for Stainless steel wire mesh instead of galvanized.

QUALITY AND SERVICE A.H.T.CO. PHILA. U.S.A. LABORATORY APPARATUS

ARTHUR H. THOMAS COMPANY

More and more laboratories rely on Thomas / Laboratory Apparatus and Reagents

VINE ST. AT 3RD • PHILADELPHIA 5, PA.

The Journal of Laboratory and Clinical Medicine

VOL. 54 AUGUST, 1959 NO. 2

CLINICAL AND EXPERIMENTAL

A METHOD FOR INCREASING THE SENSITIVITY OF THE ROSE BENGAL I^{131} LIVER FUNCTION TEST WITH THE USE OF BROMSULPHALEIN

ISMAEL MENA, M.D.,* RAYMOND KIVEL, M.D.,** PAUL MAHONEY, M.D., SHERMAN M. MELLINKOFF, M.D., AND LESLIE R. BENNETT, M.D.
LOS ANGELES, CALIF.

A TEST of hepatic cell function using tracer amounts of radioiodinated rose bengal and external scintillation detection over the liver was reported in 1955 by Taplin and associates.[5] In a continuation of his animal experiments Taplin has reported that in rabbits with minimum chloroform injury in which no defect could be shown in the liver uptake of tracer amounts of rose bengal I^{131}, a marked impairment of the uptake of the radioactive dye could be induced by administration of a stressing dose of Bromsulphalein just before the rose bengal I^{131}. The same procedure had no effect on the uptake by normal rabbit liver.

The phenomenon of interference of sodium dehydrocholate with the clearance of Bromsulphalein (BSP) from the blood was pointed out by Cantarow and Wirts.[1] Mendeloff and associates[2] reported that BSP reduced the removal from the blood of rose bengal by the normal liver; bilirubin, rose bengal, and fluorescein had no effect on BSP removal, however. Cohen and associates[3] showed that galactose also had no effect on BSP removal. In a later report[4] they confirmed the fact that BSP markedly interfered with rose bengal clearance and showed that the latter had only a minor effect on BSP clearance.

In working with Westover's[6] modification of the I^{131} rose bengal uptake test in human subjects we observed that patients with mild but proved liver

From the Departments of Radiology and Medicine, University of California Medical Center, Los Angeles, Calif.
Received for publication Dec. 11, 1958.
*Fellow, the Henry L. and Grace Doherty Charitable Foundation, Inc.
**Fellow in Gastroenterology, United States Public Health Service.

disease would often have uptake values that were within the normal range, usually low normal values.

The demonstrated interference of BSP with rose bengal uptake was used in the present study for the following purposes: (1) To confirm the BSP inhibition of rose bengal uptakes in human beings when tracer amounts of rose bengal are used; (2) to evaluate this "stress" test in the detection of border line liver disease; and (3) to explore further the mechanism of the interference process.

MATERIALS AND METHOD

The determination of the uptake rate of rose bengal I^{131} is performed in accordance with the method of Westover and co-workers.[6] A probe is placed over the eighth right intercostal space on the anterior axillary line. A sodium iodide scintillation crystal, 1½ by ¾ inch, is set at 6 cm. from the skin. The scintillation probe is then connected to a precision ratemeter that feeds its information to an Esterline-Angus recorder. The ratemeter is set at 10 K and at a 10-second time constant, and the recording paper starts to record background at a speed of 2 cm. per minute. At this point an intravenous injection of RISA (radioactive iodinated human serum albumin) is given to the fasting individual. The dose is in the amount of 0.03 μc/Kg. and gives rise to a peak of radioactivity over the liver followed by a plateau (Fig. 1). Westover interprets this plateau as reflecting the size of the vascular pool in the liver. Two minutes later rose bengal I^{131} 0.03 μc/Kg. is injected intravenously.

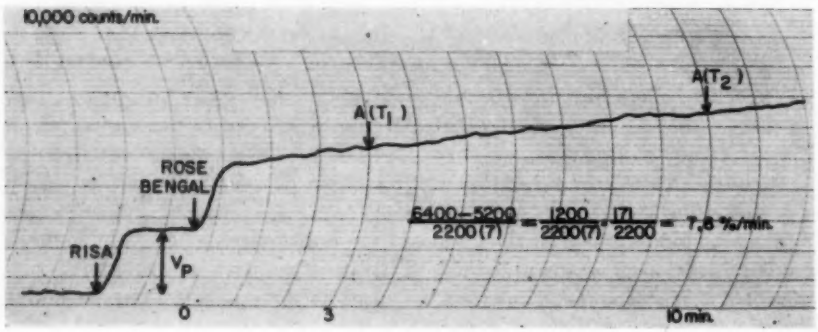


Fig. 1.—Liver uptake of rose bengal I^{131}.

The curve again rises rapidly as the tagged dye enters the liver and continues to rise as the cellular uptake of rose bengal progresses. The recording is continued for 10 minutes, thus obtaining a rate of rose bengal liver uptake.* The rate of rose bengal uptake is determined per minute, and the ratio of this value to the counts per minute obtained with RISA gives normal values of 11 to 20 per cent per minute. This way of reporting the rate of uptake, in accordance with Westover's method, makes it possible to ignore changes in the amount of rose bengal injected and in the geometrical conditions, e.g., body configurations, thickness of body wall, etc.

In this study 3 groups of individuals were used. The first group was comprised of persons with normal liver function and no history of liver disease. The second included

*Fig. 1 is labeled to clarify the method of calculation:

$$Q = \frac{A\ (t_2) - A\ (t_1)}{V_p\ (t_2 - t_1)} = \text{per cent uptake per minute}$$

where $A(t_1)$ = counts per minute at 3 minutes after injection of rose bengal.
$A(t_2)$ = counts per minute at 10 minutes after injection of rose bengal.
V_p = counts per minute; activity entering the liver is a measure of liver vascularity.

patients with mild chronic liver disease, slightly abnormal retention of Bromsulphalein after a 5 mg./Kg. dose at 45 minutes, and normal rose bengal uptakes. A third group consisted of persons who had both abnormal rose bengal and Bromsulphalein tests. These groups were divided into two series. One was tested before and after stressing with 5 mg./Kg. of Bromsulphalein, and the second after stressing with 2.5 mg./Kg. of the dye.

RESULTS

Rose bengal I^{131} liver uptake has been measured in 48 individuals who were tested before and after injection of 5 mg./Kg. of BSP. Fig. 2 shows the rose bengal uptake after BSP in 27 normal individuals that showed normal BSP blood retention at 45 minutes (0 to 3 per cent BSP retention). In all 27 cases the rose bengal uptake rate was depressed, but in 15 instances it remained in the range that would be normal without BSP injection, while in 13 instances it was depressed by the BSP injection to a level that would otherwise have been abnormal. The mean of rose bengal uptake was 10.2 per cent, standard deviation 3.1 per cent.

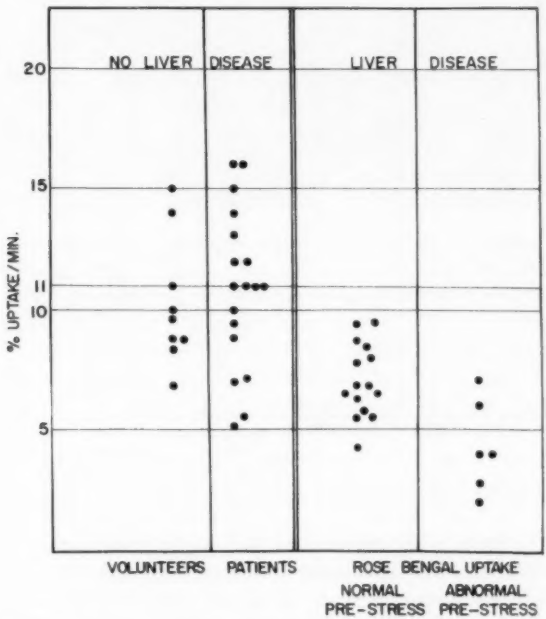


Fig. 2.—Uptake of rose bengal I^{131} after the administration of 5 mg./Kg. of BSP.

Twenty-one patients with portal cirrhosis or chronic hepatitis, all of them with abnormal BSP retention at 45 minutes, were tested in the same way. Fifteen of these patients had a normal base-line rose bengal uptake before BSP injection. After injection of 5 mg./Kg. of BSP, the uptake of rose bengal in all patients was depressed to the abnormal range. Mean was 6.3 per cent, standard deviation 2.1 per cent.

Effect of 2.5 mg./Kg. of BSP on Rose Bengal Uptake.—In an attempt to determine the critical amount of BSP that would separate the normal from the abnormal group, the effects of 2.5 mg./Kg. of BSP were studied in the same 10-minute period after injection.

Fifty-six additional individuals were tested, i.e., not the same individuals used in the previous study. Fig. 3 summarizes the results in this group. Thirty-one normal individuals were studied. Twenty-eight remained in the normal rose bengal uptake range after BSP injection, and 3 were depressed to a borderline abnormal range (10.5 per cent). The mean was 13.2 per cent and standard deviation 2.1 per cent.

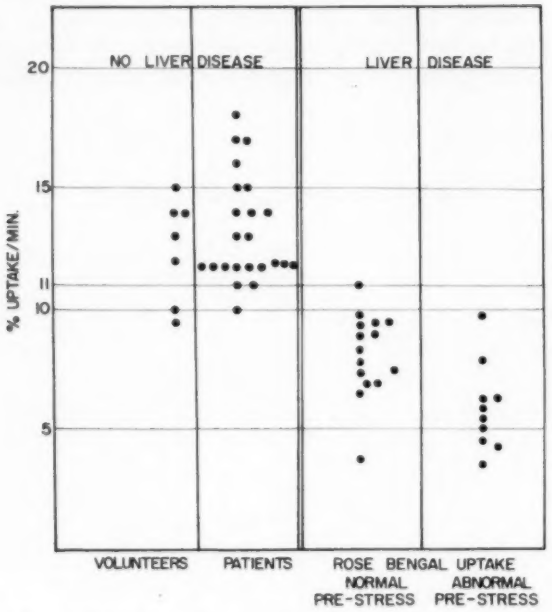


Fig. 3.—Uptake of rose bengal I^{131} after the administration of 2.5 mg./Kg. of BSP.

Twenty-five patients with cirrhosis or chronic hepatitis were tested. Sixteen showed normal base-line rose bengal uptake, but after 2.5 mg./Kg. BSP was injected all of these patients were depressed to an abnormal range. Nine patients started in the borderline abnormal range and depressed still further after the BSP injection. Mean was 6.9 per cent, standard deviation 2.1 per cent.

DISCUSSION

The results show clearly that hepatic rose bengal uptake as determined by the method reported here without BSP injection can be normal in the presence of minimal liver disease. Fifteen of 21 patients with abnormal BSP retention in the first series and 16 of 25 patients in the second series had normal rose

bengal uptakes. This incidence, of course, was high only because such patients were deliberately sought for the purpose of this experiment and were selected from a much larger number in which this discrepancy between the BSP and rose bengal tests was not present. In all 15 of the Series 1 patients with abnormal BSP retention and normal rose bengal uptake, the values of the latter were depressed to an abnormal range by the injection of BSP 5 mg./Kg. This was also true for patients with minimal liver disease and normal or borderline abnormal rose bengal uptake; rose bengal uptakes were depressed to an abnormal range by 2.5 mg./Kg. of BSP. These results indicate that in mild liver disease rose bengal uptake becomes a much more sensitive test of liver function after the injection of BSP.

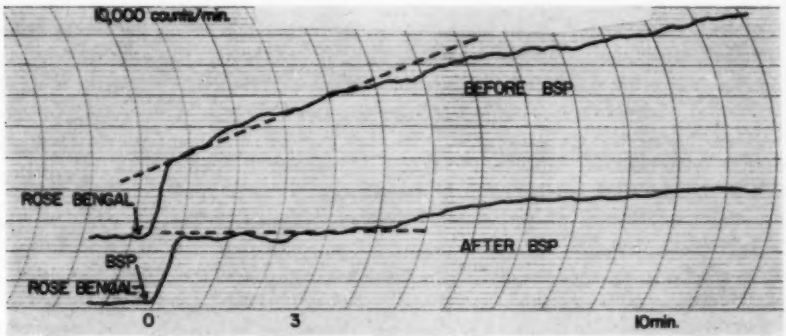


Fig. 4.—Liver uptake of rose bengal I^{131} before and after the administration of 2.5 mg./Kg. BSP.

In 28 of 31 normal individuals rose bengal uptake was normal before as well as after the injection of 2.5 mg./Kg. of BSP. In the other 3 individuals rose bengal uptake was depressed to a borderline range by this dose of BSP. The data also show that when the dose of BSP is 5 mg./Kg., most normal individuals respond with a marked depression of the rose bengal I^{131} uptake. The sensitivity of the rose bengal uptake becomes far too great to be clinically useful under these conditions, whereas a dose of 2.5 mg./Kg. greatly increases the capacity of the rose bengal uptake to distinguish individuals with normal and abnormal liver function.

This degree of sensitivity is not necessary when liver function is badly impaired.[5, 6] When hepatic damage is moderate or severe, the rose bengal uptake alone, without BSP injection, is depressed.

Whether the rose bengal uptake after BSP injection at the 2.5 mg./Kg. dosage level is generally a better index of liver function than an ordinary BSP test using 5 mg./Kg. cannot be settled by these data. A sufficient number of comparisons have not been made. The patients reported here were selected for one particular purpose, to find out whether or not the rose bengal test could be rendered more sensitive by injection of BSP when this degree of sensitivity is required. We have studied several patients, not included in this study because there is as yet an insufficient number who have been followed to certainty

of diagnosis, in whom the BSP test (5 mg./Kg.) was normal, while the rose bengal uptake after injection of BSP was definitely abnormal. One such patient was found at laparotomy two months after the tests were performed to have a liver seeded with carcinoma of the breast. More patients must be studied before any conclusions can be drawn about the relative merits of the BSP test and the rose bengal test after BSP injection.

Taplin and associates have proposed that rose bengal and BSP are cleared by the same hepatic enzyme systems.[5] Competitive inhibition could thus explain the suppression of rose bengal uptake by the BSP injection. An alternative explanation is suggested by the time relationships in the rose bengal uptake graphs after BSP injection (Fig. 4). Following the injection of 2.5 mg./Kg. of BSP in some individuals, rose bengal uptake for the first 3 or 4 minutes is decidedly lower than it is in most normal individuals injected with rose bengal alone, but subsequently the hepatic rose bengal uptake increases so rapidly that the total rose bengal uptake reaches the normal range in 10 minutes (Fig. 4). This initial depression of rose bengal uptake was followed by adequate recovery in almost all of the patients given 2.5 mg./Kg. of BSP, while relatively few patients recovered so rapidly from the depression induced by 5 mg./Kg. of BSP. Such a brief period of suppressed rose bengal uptake could be due to competitive inhibition of the enzyme system. However, the hepatic clearance of either rose bengal or BSP is dependent upon two factors: (1) the ability of the liver cells to extract the dye from the blood, and (2) the rate of blood flow. It is, therefore, also possible that the depression of rose bengal uptake induced by BSP injections was due to a temporary alteration in hepatic blood flow. The latter possibility might more easily explain the relatively great suppression of rose bengal uptake by BSP for only a few minutes with such prompt and complete recovery. This alternative also seems more compatible with the discrepancy in dosage between the competing dyes. So marked a depression of uptake of such a minimal quantity of rose bengal would appear more likely to be caused by a change in blood flow than by enzymatic inhibition. The same phenomenon lasting for 10 minutes or so after the higher dose of BSP could account for the marked suppression of rose bengal uptake in normal individuals.

Whatever the explanation for BSP suppression of rose bengal uptake, it is clear that the mechanism is principally operative in the first few minutes after the injection. This rapidly changing effect is plainly reflected by the rose bengal tracer technique, but only the ultimate effect is measured by a single 45-minute colorimetric blood determination.

SUMMARY

1. In the presence of mild liver disease the uptake of rose bengal I^{131} by the liver is within normal range.

2. A method to increase the sensitivity of this technique is described. It uses the depressing effect of BSP on the uptake of rose bengal I^{131} by the liver.

3. A dose of 5 mg./Kg. of BSP will diminish the uptake of rose bengal to an abnormal range in 100 per cent of the patients with liver disease tested, and in 48 per cent of normal controls.

4. A useful separation of abnormal patients from normal individuals can be achieved by the administration of 2.5 mg./Kg. BSP; this depresses the rose bengal uptake values of patients with liver disease, but does not affect the normal individuals.

We are indebted to Miss Jeanne Larson for her valuable technical assistance.

REFERENCES

1. Cantarow, A., and Wirts, C. W.: The Effect of Dog's Bile, Certain Bile Acids, and India Ink on Bilirubinemia and on Excretion of Bromsulfalein, Am. J. Digest. Dis. **10**: 261-266, 1943.
2. Mendeloff, A. I., Kramer, P., Ingelfinger, F. J., and Bradley, S. E.: Studies With Bromsulfalein. II. Factors Altering Its Disappearance From the Blood After a Single Intravenous Injection, Gastroenterology **13**: 222-234, 1949.
3. Cohen, E. S., Althausen, T. L., Uyeyama, K., and Treager, H.: Studies on Bromsulfalein Excretion. I. Results of Successive Daily Bromsulfalein Tests and the Effect of Galactose on Bromsulfalein Excretion, Gastroenterology **17**: 572-577, 1951.
4. Cohen, E. S., Giansiracusa, J. E., and Althausen, T. L.: Studies on Bromsulfalein Excretion. III. The Simultaneous Performance of the Bromsulfalein and Rose Bengal Excretion Tests in Individuals With Normal Hepatic Function, Gastroenterology **25**: 237-242, 1953.
5. Taplin, G. V., Meredith, O. M., and Kade, H.: Development of a Radioisotope Tracer Test for the Differential Diagnosis of Jaundice, Louisiana State M. J. **109**: 255-266, 1957.
6. Westover, J. L., Greenfield, M. A., and Norman, A.: A Clinically Useful Liver Function Test Using Rose Bengal, J. Lab. & Clin. Med. **54**: 174-177, 1959.

A CLINICALLY USEFUL LIVER FUNCTION TEST USING RADIOACTIVE ROSE BENGAL

JOSEPH L. WESTOVER, M.D., MOSES A. GREENFIELD, PH.D., AND AMOS NORMAN, PH.D.
LOS ANGELES, CALIF.

IN 1954, Taplin and co-workers initiated the use of radioiodinated rose bengal as a test of liver function. Their animal experiments revealed that the dye was removed from the blood selectively by the hepatic polygonal cells and then excreted in the bile with no reabsorption by the gastrointestinal tract. The firmly bound iodine was not taken up by the thyroid. In spite of great promise as a useful clinical procedure, the rose bengal test has not been widely accepted. Some of the reasons for this are that, in some instances, the method of extracting data has failed to yield pertinent information. Moreover, no uniform method for calculating or reporting the results has been devised. Arbitrary units have been used, and results have even been given in terms of counting rates which, obviously, cannot be duplicated on different sets of equipment.

The purpose of this paper is to present a simple, clinically useful procedure, easily calculated, which can readily be duplicated in other isotope laboratories.

MATERIALS AND METHODS

Our equipment consists of a wide angle, scintillation detector with a 1½ inch sodium iodide crystal. The crystal face is 6 cm. from the lower edge of the lead shield. A precision ratemeter, operated on a 10-second time constant and 10 K scale, is used to drive a standard Esterline-Angus recorder. The recorder is geared so that the distance between curvilinear lines on the paper represents a time interval of one minute. The recorder is operated at a faster rate than Taplin used in his early studies and gives a more detailed record of events during the crucial first few minutes. If a recorder is not available, a scaler with pre-set time is suitable. Pre-set 1-minute counts at appropriate intervals can be plotted.

For an accurate check on the dose of radioactive material, we employ a presswood syringe holder (Fig. 1). The holder has been carefully calibrated in counts per second per microcurie for 2, 5, and 10 ml. syringes. The predetermined volume of radioactive material is withdrawn. The syringe, needle, and needle-sterilizing tube are placed in the holder with the fluid column centered at the cross line. Counts are done before and after injection. After injection, the counting is done with the syringe filled with water to the initial volume. By simple subtraction, the given dose is accurately determined.*

Techniques.—With the patient in the supine position, the tube is placed anterolaterally over the liver at the level of the eighth or ninth intercostal space. A small dose of RISA

From the Department of Radiology, University of California, School of Medicine, Los Angeles, Calif.

Received for publication Dec. 11, 1958.

*This holder is used to accurately determine the administered dose in blood volume studies. It is much more satisfactory than the method of weighing the syringe before and after injection. The error in administered dose, by the old method, was found to be as much as 10 to 15 per cent.

(radioactive iodinated human serum albumin) is injected into the antecubital vein. Without removing the needle, the syringe is changed to keep the needle open with normal saline. After 2 or 3 minutes, when a suitable plateau has been recorded on the paper, the syringe is changed and rose bengal is injected. For reasons to be explained, the activity of RISA and rose bengal should be as nearly equal as possible. Our average dose of each is 2 μc in a volume of 1 to 2 c.cm. The amount required for suitable counting rates will vary with the counting efficiency of the equipment.

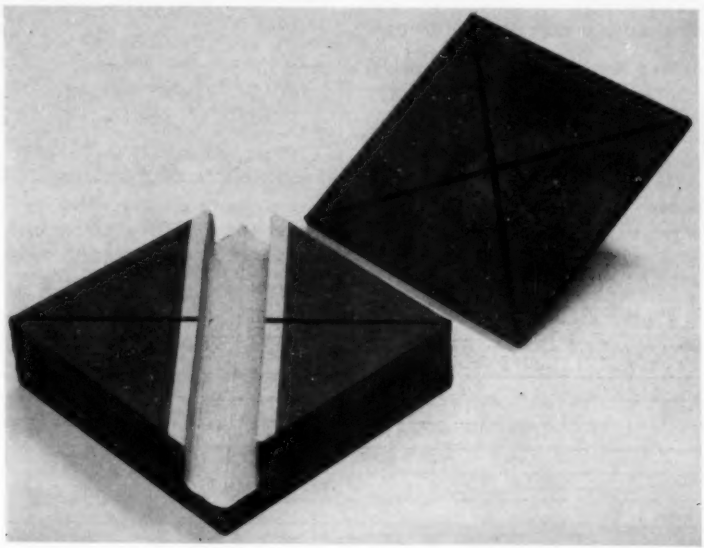


Fig. 1.—The presswood syringe holder is shown at the left. The syringe, needle, and needle-sterilizing tube are placed in the groove with the fluid column centered as the cross line. The cover is placed over the holder and the scintillation tube is centered to the cover.

Calculation.—Fig. 2 is a tracing of a normal uptake curve approximately labeled to clarify the following calculation.

$$Q = \frac{A(t_2) - A(t_1)}{V_p\ (t_2 - t_1)}$$

where $A(t_1)$ = counts per minute at 3 minutes after injection of rose bengal. $A(t_2)$ = counts per minute at 10 minutes after injection.

V_p = counts per minute of the RISA plateau. This is a measure of liver vascularity.

$\frac{A(t_2) - A(t_1)}{t_2 - t_1}$ = change in counting rate per unit time.

Q = fraction per minute of activity *in* the liver taken up by the polygonal cells.

An example from Fig. 2 follows:

$$Q = \frac{8{,}800 - 6{,}600}{2{,}400(7)} = 0.13 \text{ per minute}$$

i.e., initially, the liver is taking up rose bengal at the rate of 13 per cent per minute of the dose in the liver.

DISCUSSION

Examination of many curves reveals that the initial portion of the uptake curve is linear. From this, one can assume that excretion is negligible during

the first few minutes. This is to be expected from what is known about liver physiology. To determine the increment of rose bengal per unit time which is taken up, the level of activity entering the liver must be known. The RISA plateau gives us this activity level, V_p, and makes possible the calculation of Q. Therefore, Q is the fraction of rose bengal cleared per minute by the liver. The reason for using equal doses of activity for both RISA and rose bengal is because it simplifies the calculation of Q. Unequal doses necessitate a correction based on counting rate per microcurie.

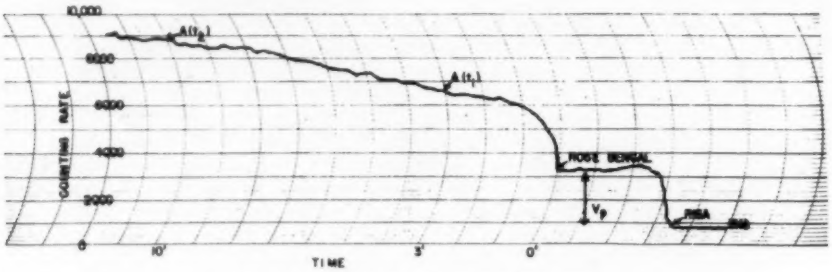


Fig. 2.—Tracing of normal uptake curve.

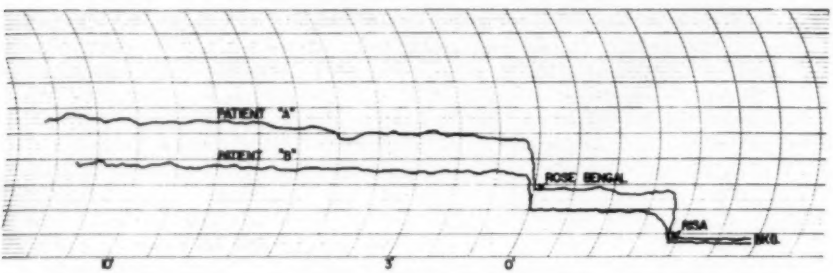


Fig. 3.—Tracings of uptake curves on two patients to demonstrate the usefulness of the RISA modification. Patient A represents a case of severe viral hepatitis; Patient B, a case of Laennec's cirrhosis. Note the difference in elevation of the RISA plateaus.

The quantity Q has a number of advantages. First, it measures a physiologic function of the liver. Second, it is independent of the isotope energy, quantity, counting geometry, or instrument calibration factors. For example, if the doses of radioactivity are doubled, A_1, A_2, and V_p will be doubled but Q will remain constant.

The RISA modification was first reported in 1955. It was instituted because in our experience, we were unable to obtain good clinical correlation with rose bengal alone, because often, as can be seen in Fig. 2, V_p cannot be determined from the initial rise in the rose bengal curve. In addition to being a convenient way to normalize the clearance rate, the determination of V_p is a separate, clinically useful test in itself. Calculations based on slope index or half-time determinations will not separate certain cases where a difference in hepatic function actually exists. Fig. 3 is an example of the uptake curves being parallel but the clearance is different.

One of the striking observations in Fig. 3 is the marked difference in hepatic vascularity, as measured by V_p. With few exceptions, a definitely decreased vascularity has been found in Laennec's cirrhosis. This finding is an immediate aid in differential diagnosis.

When repeat studies are done on the same patient, only rose bengal is used and values are computed from the original vascularity level. This avoids multiple injections of RISA and correspondingly high background levels.

This test is being used in our laboratory as the basis for further studies of uptake and excretion in the differential diagnosis of jaundice and in hepatic function responses to stress substances. As a first step, we determined the "normal" range of V_p and Q values in 40 control subjects. With our equipment, using approximately 2 μc each of RISA and rose bengal, the mean value for V_p (elevation of the RISA plateau) is 2,400 counts per minute. The range is 2,000 to 2,800 counts per minute. (Here is an unfortunate example of figures with little meaning in other laboratories.) Standard deviation reveals a value for sigma of 10 per cent of the mean. If one cares to narrow the above range, the doses can be adjusted to weight of the patient. At present, we are using 0.03 μc per kilogram of body weight.

The range of uptake, Q, for these 40 subjects is 11 to 20 per cent per minute. These values should be encountered in other isotope laboratories, since these are measurements of the function of the polygonal cells. This test is proving to be extremely valuable in following cases of viral hepatitis, as well as in the differentiation of Laennec's cirrhosis from other entities.

SUMMARY

A modified procedure is presented for obtaining the liver uptake of rose bengal. This procedure permits the easy calculation of per cent uptake per minute and is independent of such parameters as dose of isotope, counting geometry, and efficiency of counting equipment. For these reasons, the test is reproducible and clinically useful. Moreover, an index of hepatic vascularity is also obtained.

REFERENCES

1. Taplin, G. V., Meredith, O. M., Jr., and Kade, H.: University of California at Los Angeles AEP Report No. 319, 1954.
2. Taplin, G. V., Meredith, O. M., Jr., and Kade, H.: The Radioactive (I^{131}-Tagged) Rose Bengal Uptake-Excretion Test for Liver Function Using External Gamma Ray Scintillation Counting Techniques, J. Lab. & Clin. Med. **45**: 665, 1955.
3. Taplin, G. V., Meredith, O. M., Jr., Kade, H., Westover, J. L., Hansen, R. A., and Bennett, L. R.: The Radioactive (I^{131} Labelled) Rose Bengal Liver Uptake-Excretion Test. Its Use in the Evaluation of Hepatic Function, International Conference on Peaceful Uses of Atomic Energy, July 1, 1955.
4. Brown, C. H., and Glasser, O.: Radioactive (I^{131}-Tagged) Rose Bengal Liver Function Test, J. Lab. & Clin. Med. **48**: 454-462, 1956.
5. Marshall, W., and Kozoll, D. D.: Liver Uptake and Blood Clearance Studies of Radioactive (I^{131}) Rose Bengal, J. Lab. & Clin. Med. (Abst.) **48**: 924-925, 1956.
6. Lowenstein, J. M.: Quantitative Analysis of the Radioactive Rose Bengal Test of Liver Function, Clin. Res. Proc. **5**: 39-40, 1957.
7. Blahd, W. H., and Nordyke, R. A.: The Blood Disappearance of Radioactive Rose Bengal. A Rapid, Simple Test of Liver Function, Clin. Res. Proc. **5**: 40, 1957.
8. Wood, J. A., and Korst, D. R.: Clinical Studies With Radioiodine Rose Bengal Dye, Clin. Res. Proc. **5**: 210, 1957.

THE ERYTHROCYTE UPTAKE OF I^{131}-LABELED l-TRIIODOTHYRONINE AS A MEASURE OF THYROID FUNCTION

ALVIN L. URELES, M.D., AND MARTIN MURRAY, M.S.
ROCHESTER, N. Y.

RECENTLY, Hamolsky, Stein, and Freedberg[1] presented a method for studying thyroid function by utilizing the in vitro erythrocyte uptake of I^{131}-labeled l-3, 5, 3′-triiodothyronine (TRI^{131}) from human plasma. (RCU is the symbol used for the method in this paper.) Their studies indicated that the RCU could differentiate various levels of thyroid activity with certain limitations.

It was the purpose of our study to evaluate the RCU method for routine use in a thyroid laboratory.

METHODS AND MATERIALS

The method has been presented in detail by Hamolsky and associates.[1] Briefly, it consists of adding 0.1 ml. of a standard solution of TRI^{131} to 3 ml. aliquots of oxalated whole blood in a 10 ml. volumetric flask. The flasks are stoppered and shaken in a 37° C. water bath for two hours, at which time 1.0 ml. aliquots are removed and counted in a well-type scintillation counter. The aliquots are then centrifuged, the supernatant removed, and the erythrocytes washed 5 times in tenfold volumes of isotonic saline. The labeled iodine of the erythrocytes is measured, and the per cent uptake is calculated and corrected to 100 per cent hematocrit for comparative purposes. Six or more specimens may be run together. In our experience, the total time requires approximately 5 hours.

We have carried out 432 RCU determinations on 401 patients. There were 325 females, and 76 males, with ages ranging from 3 months to 80 years.

Euthyroid patients were chosen from among hospital patients or private ambulatory patients coming through our clinical laboratories at the Genesee Hospital. Hyperthyroid and hypothyroid patients were referred from our Thyroid Clinic or from private physicians. Thyroid status was determined in all cases by the clinical evaluation in addition to one or more laboratory procedures. The serum protein-bound iodine (PBI), using the Barker method,[2] was determined in 371 cases (euthyroid range 3.5 to 7.5 mg. per 100 ml.). The 24-hour radioactive I^{131} thyroidal uptake (24-hour I^{131} uptake) was determined in 81 cases (euthyroid range 17 to 50 per cent), and the basal metabolic rate (BMR) in selected instances.

RESULTS

I. Euthyroidism.—There were 222 euthyroid patients whose RCU's were between 11.5 and 18.5 per cent (averaging 14.4 per cent). The PBI was determined in 195 patients and was within the normal range in each instance.

From the Endocrine Service of the Department of Medicine, Isotope and Clinical Laboratories of the Genesee Hospital, and the Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

Received for publication Dec. 23, 1958.

There were 9 patients in whom the RCU was below 11.5 per cent and 14 cases in whom it extended above 18.5 per cent. No explanation for these values could be elicited on the basis of the available clinical and laboratory data.

Thus, of the total 245 euthyroid patients 91 per cent were within the limits of 11.5 to 18.5 per cent.

II. Hyperthyroidism.—There were 32 RCU determinations in 26 patients with varying stages of hyperthyroidism. In 6 patients whose RCU was repeated, the disease had recurred or the patient had failed to respond to treatment at the time of the repeat determination. In 29 determinations the RCU ranged from 18.5 to 32 per cent, averaging 22.7 per cent. Three patients had RCU values below 18.5 per cent.

Twenty-two of 24 patients in this hyperthyroid group had PBI levels above 8.0 μg per 100 ml. (range 8.2 to 28 μg per 100 ml., averaging 12.6 μg per 100). Two patients with nodular toxic goiter had normal PBI rates (range 5.0 and 6.2 μg per 100 ml.).

The 24-hour I^{131} uptake was above 50 per cent in all but 3 of 20 patients studied (range 50 to 88 per cent).

Therefore, the RCU (setting the hyperthyroid range at 18.5 per cent and above) failed to corroborate the diagnosis in 3 patients but appeared correct in 29 instances (91 per cent), including the two patients with nodular toxic goiter in whom the PBI was normal. The 24-hour I^{131} uptake was misleading in 3 patients, in each of whom the RCU and PBI confirmed the diagnosis.

III. Hypothyroidism.—A. There were 8 patients with hypothyroidism whose RCU ranged from 7.5 to 11 per cent, averaging 9.9 per cent. The PBI in these cases ranged from 1.4 to 3.7 μg per 100 ml., averaging 2.3 μg per 100 ml.

B. There were two hypothyroid patients with RCU's of 9.5 and 11 per cent, respectively, in whom the PBI was elevated (10.2 and 6.6 μg per 100 ml.) by cholangiographic and gall bladder contrast media 2 months and 1 month prior to study. The basal metabolic rates were –33 and –23 per cent. Both patients responded to therapy.

C. There were 10 patients who were taking 16 to 130 mg. of desiccated thyroid daily from 2 weeks to 10 years' duration. Seven had spontaneous hypothyroidism, one surgically induced, and two RAI^{131} induced hypothyroidism. They were generally improved but clinically were still hypothyroid. Their RCU's ranged from 9 to 11 per cent (averaging 10.3 per cent). Their PBI ranged from 1.4 to 3.5 μg per 100 ml. (averaging 2.2 μg per 100 ml.).

D. Two patients with hypothyroidism had RCU's of 17.6 and 14.6 per cent, respectively, and PBI's of 3.4 and 3.4 μg per 100 ml. They had symptoms and signs consistent with low thyroid function. Basal metabolic rates and 24-hour I^{131} uptakes were not done. The patients responded to treatment.

Therefore, 20 of 22 patients with hypothyroidism (91 per cent) had RCU's below 11.5 per cent and PBI's within the hypothyroid range. In 2 subjects the RCU was above 11.5 per cent with PBI less than 3.5 μg per 100 ml. In two other patients the PBI was artifactually elevated.

There were 7 additional patients in whom the RCU agreed with the clinical evaluation, although the PBI was elevated above 3.5 μg per cent. The BMR and 24-hour I^{131} uptake studies were not available for further confirmation of the diagnosis.

IV. Artifactual Elevation of the PBI Not Affecting the RCU (Table I).—There were 19 euthyroid patients whose PBI determinations were elevated above the normal range due to inorganic or organic iodides. The RCU remained in the euthyroid range (11.6 to 18.3 per cent) in all instances. Radiographic dyes, iodinated medications such as found in certain vaginal suppositories, skin preparations, and expectorant cough mixtures did not significantly alter the RCU.

TABLE I. THE EFFECT OF ORGANIC AND INORGANIC IODIDES ON THE RCU

Case 25.	A 60-year-old euthyroid man 5/16/57 gall bladder x-ray (telepaque) 5/23/57 RCU 15.6% PBI 21.5 μg/100 ml. 9/13/57 RCU 14.1% PBI 7.0 μg/100 ml.
Case 160.	A 40-year-old euthyroid woman with vaginitis treated with Floraquin 8/23/57 RCU 13.0% PBI 8.5 μg/100 ml.
Case 37.	A 26-year-old euthyroid woman with bronchitis treated with saturated solution of potassium iodide 5/29/57 RCU 16.0%
Case 235.	A 38-year-old hypothyroid woman received intravenous cholangiogram 1 month prior to study 10/29/57 RCU 10.1% PBI 9.7 μg/100ml.

Two hypothyroid patients whose blood was contaminated with organic iodine containing contract media were mentioned previously in Section III, B.

V. Biologic Alterations of the RCU (Table II).—A. The augmenting effects of liver disease, hypoalbuminemia, and albuminuric renal disease on the RCU have been described.[1]

In this study, 18 euthyroid patients with RCU elevations above 18.5 per cent had long-standing congestive heart failure associated with hepatomegaly, acute or chronic liver disease associated with or without demonstrable decreases in serum proteins, or renal disease associated with moderate to severe albuminuria.

B. The effects of pregnancy on the RCU and PBI have been documented.[1, 3, 4] In 6 of 8 pregnant patients the RCU was below 11.5 per cent (average 10.3 per cent) and PBI was elevated (average 7.9 μg per 100 ml). One of the two pregnant patients in whom the RCU was above 11.5 was a patient with a total thyroidectomy who was taking 325 mg. desiccated thyroid per day and whose thyroxine binding sites presumably were saturated (see discussion). The second patient, who had two previous spontaneous abortions, was 2 months pregnant. The RCU was 14.2 per cent. The PBI was only 5.7 μg per 100 ml. The relationship of spontaneous abortion to a failure of the PBI to become elevated in pregnancy has been noted.[3]

Patients undergoing estrogen therapy had similarly depressed RCU values.

TABLE II. BIOLOGIC ALTERATIONS IN THE RCU

DIAGNOSIS	CASE NO.	RCU (%)	PBI (μg/%)	24 HOUR RAI (% UPTAKE)	COMMENT
Congestive heart failure with hepatomegaly	144	27.0	4.6	36	Severe decompensation
		25.0	4.6	--	After compensation
	219	21.2	5.5	--	Ascites
	362	20.0	--	--	Failure, infarction
	40	19.9	6.4	--	Failure, rapid auricular fibrillation
Liver disease with or without decreased serum albumin	261	27.3	--	--	Acute and chronic liver disease
	394	23.4	6.0	37	Alcoholic cirrhosis
	53	22.0	4.9	--	Chronic hepatitis
	147	21.7	9.6	--	Ulcerative colitis
	1	21.5	--	15	Malnutrition
	414	19.8	3.9	--	Low serum albumin
	88	18.5	--	--	Alcoholic cirrhosis
	280	18.5	4.9	--	Hepatitis, subacute
Renal disease	200	17.1	5.8	--	Nephrotic syndrome
	315	19.3	--	--	Diabetic nephropathy
	36	20.0	4.5	--	Diabetic nephropathy
	121	20.5	4.4	33	Diabetic nephropathy
	61	22.5	5.1	--	Albuminuria, ? etiology
Pregnancy	245	7.1	7.0	--	
	69	8.1	7.8	--	On 130 mg. thyroid
	256	8.5	8.8	--	
	48	8.8	7.8	--	On 130 mg. thyroid
	238	9.3	8.6	--	
	234	11.3	7.9	--	On 130 mg. thyroid
	272	13.0	--	--	On 325 mg. thyroid
	268	14.2	5.7	--	Spontaneous abortion

C. Of 12 patients previously treated for hyperthyroidism, 5 had RCU's above 18.5 per cent, 4 were between 17 and 18.3 per cent, and 2 who were hypothyroid at the time of study had values below 11.5 per cent.

VI. Correlation of the RCU With Clinical Diagnosis, PBI, and 24-Hour I^{131} Uptake.—Eighty-one patients had RCU's, PBI's, and 24-hour I^{131} uptakes determined jointly. These determinations were correlated with the clinical diagnosis.

Two of four patients with hypothyroidism had studies in complete agreement. In the third, only the RCU was confirmatory. The PBI and 24-hour I^{131} uptake were disturbed by organic iodide and a hyperplastic gland. The fourth patient, with chronic potassium thiocyanate poisoning, had an elevated RCU and 24-hour I^{131} uptake in contrast to a low PBI which was consistent with the clinical state of myxedema.

In 34 of 57 euthyroid patients, the RCU, PBI, and 24-hour I^{131} uptakes agreed with the clinical diagnosis. In 12 patients, the RCU was disturbed by the biologically altering factors noted in Section V, A, whereas the PBI and 24-hour I^{131} remained unaffected. In 5 patients in whom the PBI was elevated by organic iodides, the RCU and 24-hour I^{131} uptake were unaffected. The 24-hour I^{131} uptake was elevated by a hyperplastic goiter in 3 patients and in one patient was depressed by potassium iodide. Two patients with hyperplasia (postthyroideetomy) had elevated RCU's and 24-hour I^{131} uptakes, whereas

the PBI was correctly euthyroid. In one patient, the 24-hour I^{131} uptake was normal in the presence of a RCU disturbed by liver disease, and a PBI altered by organic iodides.

There were 20 patients with hyperthyroidism. Thirteen had RCU's, PBI's, and 24-hour I^{131} uptakes in agreement. In 3 patients with congestive heart failure, diffuse toxic goiter, and nodular toxic goiter, the 24-hour I^{131} uptake failed to rise to hyperthyroid levels. In 3 other patients with recurrent, diffuse, and nodular toxic goiter, the RCU failed to reflect the disorder. Finally, there was one patient with nodular toxic goiter in whom the PBI failed to indicate the clinical state.

Thus, in 81 patients in whom all determinations were made, there was a positive correlation of the 3 laboratory procedures with the clinical state in 49 cases (60.5 per cent). The RCU correlated in 62 patients (76.5 per cent), the PBI in 73 (90.1 per cent), and the 24-hour I^{131} uptake in 71 (88 per cent).

It should be noted that in 12 patients in whom the RCU did not agree with the clinical state, the patients were euthyroid with associated diseases known to give elevated RCU's.

In patients whose RCU, PBI, or 24-hour I^{131} uptake did not agree with the clinical state, the discrepancy usually was associated with some biologic or chemical interference in which the diagnosis could be salvaged by one or both of the other determinations.

TABLE III. CASES TREATED WITH DESICCATED THYROID OR TRIIODOTHYRONINE (TRI)

CASE NO.	THYROID (MG./DAY)	DURATION (YEARS)	RCU (%)	PBI (μg %)	COMMENT
Group A					
66	195	10	17.3	4.1	Hypopituitarism
338	65	15	17.2	4.2	Formerly hypothyroid
185	100	0.1	15.8	--	Hypothyroid 2 mo. prior (RCU 11.4, PBI 1.7 μg %)
274	25 μg TRI 130	0.1	12.3	1.4	Hypothyroid 2 mo. prior (RCU 10.3 %, PBI 1.7 μg %)
93	65 195	3 0.2	13.6 13.9	5.0 2.9	When thyroid increased PBI decreased but RCU was the same
221	75 μg TRI	0.5	17.5	3.5	Euthyroid
413	50 μg TRI	0.5	12.8	1.0	Euthyroid
Group B					
260	100	1	18.1	3.0	Formerly toxic, now euthyroid
35	130	2	16.3	3.2	
223	100	0.5	12.7	2.4	
Group C					
384	260	1	18.2	4.8	Euthyroid
278	65	1	18.2	5.3	Euthyroid
358	162	1	17.6	5.0	Euthyroid
271	65	1	15.4	5.6	Euthyroid
203	130	1	15.1	5.5	Euthyroid
258	100	1	13.9	6.0	Euthyroid
284	260	2	12.9	6.7	Euthyroid

VII. Treatment With Desiccated Thyroid or Triiodothyronine.—Table III presents 17 patients who were treated with desiccated thyroid or triiodothyronine. All patients were euthyroid at the time of the determination.

Patients in Group A had previously been spontaneously hypothyroid and were corrected to euthyroidism. Patients in Group B were formerly hyperthyroid, became hypothyroid, and were corrected to euthyroidism. Patients in Group C were all euthyroid women with menstrual irregularities or varying degrees of infertility. In all groups the RCU was within the euthyroid range, although the PBI in Group B (cases that had been formerly hypothyroid) continued to be depressed. Patients receiving triiodothyronine had normal RCU's in spite of low PBI's.

In the 7 euthyroid women (Group C) there appeared to be an inverse relation of the RCU to the PBI within their normal ranges, independent of the dosage of desiccated thyroid.

DISCUSSION

The localization of the thyroid hormone-plasma protein complex in the inter-alpha globulin zone by paper electrophoresis at pH 7.4 and 8.6 by Gordon and associates,[5] Larson and co-workers,[6] and Robbins and Rall[7] stimulated investigation of thyroxine transport. In vitro experiments revealed a high specificity of the alpha globulin site for thyroxine which appeared to be not only more firmly bound than triiodothyronine but able to displace the latter.[8] Further studies disclosed that in various thyroid states the amount of added thyroxine bound by the thyroid-binding globulins varied inversely with thyroid function[9] suggesting that the hyperthyroid thyroid-binding globulins are relatively thyroxine saturated as compared with those of a hypothyroid patient.

Experiments conducted by Hamolsky and Freedberg[10] demonstrated that the in vitro uptake of TRI^{131} by rat diaphragm was increased from thyrotoxic plasma in contrast to euthyroid plasma. Further studies by this group utilizing the in vitro erythrocyte uptake of TRI^{131} from plasma gave support to the importance of the plasma protein thyroid hormone complex in the differentiation between hyperthyroid and euthyroid states. By a series of criscross experiments they demonstrated that euthyroid erythrocytes incubated with hyperthyroid plasma would have RCU's in the hyperthyroid range.[11]

Crispell and associates,[12] using the RCU method, demonstrated that l-TRI^{131} has a consistently higher uptake than l-thyroxine^{131} and noted that the presence of plasma markedly decreased the uptake.

It would appear that the RCU reflects the relative competition between the erythrocyte binding properties and the protein binding properties for l-TRI^{131} and that this is influenced by the degree of thyroxine saturation on the thyroid-binding globulins. That other chemical constituents can disturb this relationship has been observed by the effects of estrogen, pregnancy, Dicumarol, diseases of the liver, and kidney,[1] and by trypan blue and Evans blue.[13]

Hamolsky's group found the RCU euthyroid range predominantly between 10.3 and 17.0 per cent. In our series the range was 11.5 to 18.5 per cent. The

reasons for this shift are not apparent although our method differed in the use of volumetric rather than Erlenmeyer flasks. Determinations in which incubations were carried out in open flasks were significantly depressed and led to false values. All data were collected on blood oxalated in a standard potassium ammonium oxalate mixture; however, in 141 simultaneous determinations using potassium oxalate alone the standard error was negligible.

The relative freedom of the RCU from interference by inorganic and organic iodides probably reflects their inability to displace l-TRI^{131} from its binding sites. That both the thyroidal uptake of I^{131} and the PBI may be rendered useless by the inorganic and organic iodides appears to confer particular virtue on the RCU in these situations.

The failure of the RCU to fall in certain euthyroid or hypothyroid cases of previously treated hyperthyroidism is striking and suggests some qualitative change in the thyroid-binding globulins in spite of normal or low PBI. Two of our cases had elevated RCU's 5 years after a subtotal thyroidectomy for diffuse toxic goiter. Hamolsky and associates report 4 cases with persistent elevations one year after attaining a euthyroid or hypothyroid state.[1]

The nature of the alteration in the RCU seen in many of our cases with congestive heart failure with hepatomegaly is not yet clear and may limit its use in severe heart disease associated with hyperthyroidism.

Paper electrophoretic studies that we have made on the distribution of the l-TRI^{131} in the supernatant plasma indicate that significant quantities (approximately 25 per cent) appear on the albumin fraction. The RCU was elevated in patients whose quantitative electrophoretic protein patterns revealed decreases principally in the albumin fraction. Although decreases in the PBI have been noted in some disorders with severe hypoalbuminemia[14] all PBI's in our patient were within the normal range.

The low RCU in pregnancy probably reflects the increased binding capacity of the thyroid-binding globulins demonstrated by Dowling, Freinkel, and Ingbar,[15] the shift of the l-TRI^{131} being away from the erythrocyte. This can occur even though the patient is on desiccated thyroid as noted in Table II. When oral medication is increased to higher levels, as in case 272 (325 mg.), the thyroid-binding proteins are finally near saturation and the l-TRI^{131} shifts toward the erythrocyte. This action of oral thyroid probably accounts for the values seen in two cases of hypothyroidism noted in Table III.

The depressing effect of triiodothyronine on the protein-bound iodine has been noted by others[16] and is seen in Table III (Cases 274, 221, and 413). The RCU in these cases was normal suggesting that l-TRI^{131} could still bind effectively to the thyroid-binding proteins.

In comparing this test with the PBI and 24-hour I^{131} uptake it should be pointed out that final evaluation must await more precise knowledge of those factors that interfere with the RCU. As our experience with thyroid tests increases it is clear that no single test will suffice for all thyroid problems.

SUMMARY AND CONCLUSION

1. Four hundred and thirty-two erythrocyte uptake (RCU) determinations were performed on 401 patients utilizing the method described by Hamolsky, Stein and Freedberg.

2. In diagnostic studies 91 per cent of 222 euthyroid patients had erythrocyte uptake values within the limits of 11.5 to 18.5 per cent; of 32 hyperthyroid determinations, 91 per cent had values above 18.5 per cent; and 91 per cent of 22 hypothyroid patients had RCU's below 11.5 per cent.

3. There were 21 patients in whom the erythrocyte uptake determination revealed the correct thyroid status, in spite of gross contamination of the protein-bound iodine by inorganic or organic iodides.

4. Conditions under which the RCU is altered are reviewed. This study demonstrates that the erythrocyte uptake is a useful adjunct in the study of thyroid disorders. As with other tests of thyroid function, its limitations must be further explored and understood in order to place its value in proper perspective.

REFERENCES

1. Hamolsky, M., Stein, M., and Freedberg, A. S.: The Thyroid Hormone-Plasma Protein Complex in Man. II. A New In Vitro Method for Study of "Uptake" of Labelled Hormonal Components by Human Erythrocytes, J. Clin. Endocrinol. & Metab. **17**: 33, 1957.
2. Barker, S. B., Humphrey, M. J., and Soley, M. H.: Clinical Determination of Protein-Bound Iodine, J. Clin. Invest. **30**: 55, 1951.
3. Heineman, M., Johnson, C. E., and Man, E. B.: The Serum Precipitable Iodine in Pregnancy, J. Clin. Invest. **27**: 91, 1948.
4. Dowling, J. T., Freinkel, N., and Ingbar, S. H.: Thyroxinebinding by Sera of Pregnant Women, J. Clin. Endocrinol. & Metab. **16**: 280, 1956.
5. Gordon, A. H., Gross, J., O'Connor, D., and Pitt-Rivers, R.: Nature of the Circulating Thyroid Hormone-Plasma Protein Complex, Nature **169**: 19, 1952.
6. Larson, F., Deiss, W. P., and Albright, E. C.: Localization of Protein-Bound Radioactive Iodine by Filter Paper Electrophoreis, Science **115**: 626, 1952.
7. Robbins, J., and Rall, J. E.: Zone Electrophoresis in Filter Paper of Serum I^{131} After Radioiodide Administration, Proc. Soc. Exper. Biol. & Med. **81**: 530, 1952.
8. Deiss, W. P., Albright, E. C., and Larson, F. C.: Comparison of In Vitro Serum Protein Binding of Thyroxin and Triiodothyronine, Proc. Soc. Exper. Biol. & Med. **84**: 513, 1953.
9. Albright, E. C., Larson, F. C., and Deiss, W.: Thyroxine Binding Capacity of Serum Alpha Globulin in Hypothyroid, Euthyroid, and Hyperthyroid Subjects, J. Clin. Invest. **34**: 44, 1955.
10. Hamolsky, M. W., and Freedberg, A. S.: The Circulating Thyroid Hormone-Plasma Protein Complex in Euthyroid and Hyperthyroid Subjects; Differences in Rate of Disappearance in Dogs and "Uptake" by Rat Diaphragm, Tr. Am. Goiter A., pp. 269-274, 1954.
11. Hamolsky, M. W.: The Plasma Protein-Thyroid Hormone Complex in Thyrotoxicosis Vs. Euthyroidism in Man, J. Clin. Invest. (Abst.) **34**: 914, 1955.
12. Crispell, K. R., Kahana, S., and Hyer, H.: The Effect of Plasma on the In Vitro Uptake or Binding by Human Red Cells of Radioactive I^{131} Labeled l-Thyroxine and l-Triiodothyronine, J. Clin. Invest. **35**: 121, 1956.
13. Crispell, K. R., Coleman, J., and Hyer, H.: Factors Affecting the Binding Capacity of Human Erythrocytes for I^{131} Labeled l-thyroxine and l-Triiodothyronine, J. Clin. Endocrinol. & Metab. **17**: 1305, 1957.
14. Peters, J. P., and Man, E. B.: The Relation of Albumin to Precipitable Iodine of Serum, J. Clin. Invest. **27**: 397, 1948.
15. Dowling, J. T., Freinkel, N., and Ingbar, S. H.: Thyroxinebinding by Sera of Pregnant Women, Newborn Infants, and Women With Spontaneous Abortion, J. Clin. Invest. **35**: 1263, 1956.
16. Star, P., Snipes, G., and Liebhold-Schueck, R.: Biologic Effects of Triiodothyronine in Human Subjects, J. Clin. Endocrinol. & Metab. **15**: 98, 1955.

A NOTE CONCERNING SOURCES OF VARIANCE IN DETERMINATIONS OF HUMAN PLASMA VOLUME

JOHN E. OVERALL, PH.D.,* AND CLYDE M. WILLIAMS, M.D., PH.D.
CHAPEL HILL, N. C., AND PITTSBURGH, PA.

MANY investigators have compared radioisotope techniques for the estimations of human plasma volume with traditional dye methods. The purpose of this communication is to indicate that application of the various techniques is not yet standardized; different techniques actually measure different things when employed by a single investigator, and the same techniques yield significantly different results in the hands of different investigators.

1. Components of Variance in a Single Study.—Previous comparisons of the Evans blue dye method and the radioiodinated human serum albumin (RISA) method have *assumed* that both techniques estimate plasma volume and have questioned whether one technique produces consistently greater or smaller estimates. If, however, the two techniques actually measure different things, then one technique might yield consistently higher estimates for the plasma volume of one individual whereas the other technique would yield consistently higher estimates for another individual.

Because of their especially adequate experimental design, a study by Zipf and associates[1] provides data from which answers to these questions can be determined. Solutions of RISA and Evans blue dye were injected consecutively through the same needle. Blood samples were drawn without stasis at 15 and 20 minutes postinjection. Radioactivity assay and optical densities of dye-tinted plasma were determined and estimates of plasma volume calculated. Paired 15- and 20-minute estimates by both the dye and isotope methods were reported for a total of 29 individuals. Although both direct and indirect assays for radioactivity were reported, the present analysis involves data from the direct method only.

The 15- and 20-minute data for the 29 subjects have been subjected to a standard three-way analysis of variance. Answers to the following questions are provided by this analysis. (1) Do the two techniques (as employed by the original investigators) yield estimates which differ significantly on the average? (2) Is there a consistent difference between 15- and 20-minute estimates, i.e., are absolute magnitudes of plasma volume determinations

From the Psychometric Laboratory of the University of North Carolina, Chapel Hill, N. C., and the Radioisotope Service of the Veterans Administration Hospital, Pittsburgh, Pa.
Received for publication Jan. 5, 1959.

*National Science Foundation Postdoctoral Fellow in the Psychometric Laboratory at the University of North Carolina. This author is indebted to the National Science Foundation and to Dr. Lyle V. Jones, for making this collaboration possible.

affected by small differences in time interval between injection and sampling within the 15- to 20-minute range? (3) Over and above all differences due to errors of measurement, time intervals, and methods of determination, are there consistent differences between plasma volume estimates for different individuals? (4) Most important, for the present discussion, does the *relative* magnitude of plasma volume determinations by the dye and RISA techniques depend upon characteristics *within* the individual subject?

TABLE I. SUMMARY OF ANALYSIS OF VARIANCE COMPARING REPEATED ESTIMATES OF PLASMA VOLUME BASED UPON EVANS BLUE DYE AND RISA TECHNIQUES (DATA FROM ZIPF AND CO-WORKERS[1])

SOURCE	SS	df	MS	F	p
Between individuals	38331298	28	1368975	7.377	< 0.001
Between methods	18485674	1	18485674	32.63	< 0.001
Between replications	605816	1	605816	3.265	N.S.
Methods × replications	538427	1	538427	2.907	N.S.
Individuals × methods	15860718	28	566454	3.058	< 0.005
Individuals × replications	5195949	28	185570	1.002	N.S.
Individuals × methods × replications	5186035	28	185216		
Total	84203917	115			

A summary of the analysis of variance is presented in Table I. The first fact that is apparent is that *none* of the tests involving replication effects are significant. This result supports the conclusion by Zipf and co-workers that no change affected plasma volume estimates during the 15- to 20-minute postinjection period. Small differences in time between injection and sampling apparently do not influence plasma volume determinations by either of the methods.

The highly significant interaction between individuals and methods indicates that it is extremely unlikely ($p < 0.005$) that the dye method and the RISA method were actually measuring the same characteristics of the individuals. This fact, statistically verified in this analysis, is apparent from casual inspection of the data presented by Zipf and associates. Estimates of plasma volume are consistently larger for some individuals when determinations are based upon one technique, whereas estimates based upon the other technique are consistently larger for other individuals. It must be concluded that the dye and RISA techniques measure, at least to a statistically significant degree, different characteristics within the same individual. Thus, one or both of the methods is not an unbiased estimator of true plasma volume. Further investigation into the source of this bias is suggested and can be accomplished by comparing individuals who are characteristically higher by one method with those characteristically higher by the other method.

The analysis of variance indicates differences between individuals which are consistent across the two methods of estimation, i.e., in spite of the fact that the two techniques measure somewhat different characteristics of the individual, there is significant agreement between estimates based upon the two methods. The "component of variance" associated with differences between individual subjects may be considered an estimate of the relative

contribution of differences between individuals which are *consistent* across methods of estimating plasma volume, whereas the "component of variance" associated with the interaction between individuals and methods may be considered an estimate of the relative contribution of differences between individuals which are *not consistent* from one method of measurement to the other. A comparison of the two "components of variance" should provide an indication of the extent to which the dye method and RISA method, as employed by Zipf and associates, fail to measure the same characteristics of the individual. The "component of variance" associated with differences between individuals which are independent of the method of measurement is 295851, and the "component of variance" associated with differences between individuals which depend upon the method of estimation is 190619. Thus, variation in plasma volume estimates which results from the fact that the two methods actually measure somewhat different characteristics of the same individual is observed to be almost two-thirds as great as variation resulting from average differences between individuals.

2. Components of Variance Between Comparable Studies.—Inkley, Brooks, and Krieger[2] derived paired estimates of plasma volume for 47 individuals 10 minutes following injection of Evans blue dye and RISA. They concluded that their data supported the assumption of no difference between the two sets of estimates. However, if a statistical analysis appropriate for the correlated data is employed, estimates derived from the dye method in their study are found to be significantly *smaller* than estimates based upon the RISA method. A summary of the analysis of variance with accompanying probability levels is presented in Table II.

TABLE II. SUMMARY OF ANALYSIS OF VARIANCE FOR DATA FROM INKLEY AND ASSOCIATES COMPARING ESTIMATES OF PLASMA VOLUME BASED UPON TWO METHODS

SOURCE OF VARIANCE	SS	df	MS	F	p
Difference between individuals	19014258	46	413353	18.26	< 0.001
Difference between methods	93713	1	93713	4.14	< 0.05
Interaction of individuals + methods	1041288	46	22637		
Total	20149259	93			

Zipf and co-workers injected RISA and Evans blue dye consecutively and estimated plasma volumes 15 minutes postinjection for 31 individuals. In their data, estimates of plasma volume derived from the dye technique are significantly *larger* than those based upon the RISA method. A summary of the analysis of variance computed from the 15-minute postinjection values is presented in Table III with the accompanying probability levels.

If the individuals in the two experiments can be assumed to be sampled from the same human population, it is possible to compare the two experiments directly. Both Inkley and co-workers and Zipf and associates present body weights for all individuals in their studies. The mean body weight for all individuals in Inkley's sample was 69.29 Kg. and the mean weight for all individuals in Zipf's sample was 68.32 Kg. Differences *greater* than this would

be expected almost 70 per cent of the time by chance alone. In light of the recognized regression of plasma volume upon body weight it appears reasonable to assume that the subjects in the two studies belonged to the same human population.

TABLE III. SUMMARY OF ANALYSIS OF VARIANCE FOR DATA FROM ZIPF AND ASSOCIATES. COMPARISON OF PLASMA VOLUME ESTIMATES BASED UPON TWO METHODS AT 15 MINUTES POSTINJECTION

SOURCE OF VARIANCE	SS	df	MS	F	p
Difference between individuals	23734212	30	791140	2.53	< 0.01
Difference between methods	5896361	1	5896361	18.82	< 0.001
Interaction of individuals × methods	9394105	30	313137		
Total	39024678	61			

Table IV presents a summary of the over-all analysis of variance comparing plasma volume determinations from Inkley's study with those from the study by Zipf. The highly significant interaction between studies and methods of estimation indicates that the difference in results obtained from the two methods is not constant from one group of investigators to the next. The probability that the two groups of investigators were measuring the same thing in the same way is less than 0.001!

TABLE IV. SUMMARY OF ANALYSIS OF VARIANCE COMPARING DATA FROM INKLEY AND CO-WORKERS WITH THAT FROM ZIPF AND ASSOCIATES

SOURCE OF VARIANCE	SS	df	MS	F	p
Between individuals	47121634	77			
Difference between studies	4373163	1	4373163	7.77	< 0.01
Error (between individuals)	42748471	76	562480		
Within individuals	16425466	78			
Difference between methods	1672350	1	1672350	12.18	< 0.001
Interaction of studies × methods	4317724	1	4317724	31.45	< 0.001
Error (within individuals)	10435392	76	137308		
Total	63547100	155			

Inkley found RISA estimates to be larger, and Zipf found Evans blue dye estimates to be larger. The highly significant interaction between studies and techniques indicates that the question of which technique produces the larger estimates is best answered by saying that it depends upon who is employing the techniques. Statistical analysis of the data from Inkley and co-workers indicates that the probability is greater than 0.95 that if the same investigators were to repeat their study using the same techniques they would again find the RISA method to result in larger estimates. It is even more likely, the probability being greater than 0.999, that Zipf and associates would again find the dye method to produce the larger estimate of plasma volume. Thus, the dye method sometimes results in higher estimates, and an RISA method sometimes results in higher estimates; a major portion of this variation arises from differences in the way different technicians employ the methods.

The analysis of variance presented in Table IV indicates another kind of "technician variance" in plasma volume estimation. Although the mean body weights for subjects in the Inkley and Zipf studies were very nearly equal and it is reasonable to assume that the subjects in each sample were drawn from the same general "plasma volume population," the mean plasma volume estimated at 15 minutes postinjection by both methods in the Zipf study was 3,101 ml. while the mean plasma volume estimated by both methods in the Inkley study was only 2,758 ml. This difference in average magnitude of estimates is so great that it could have resulted from sampling error with a probability less than 0.01. It is concluded that the methods employed produced different results in the hands of different investigators.

3. Estimation of Plasma Volume in Normal Individuals.—Inkley and his co-workers have contributed substantially to the clinical usefulness of plasma volume determinations by demonstrating the significant regression of plasma volume upon certain easily obtained physical measurements. When these measurements are taken into consideration the expected range of normal variation in plasma volume is sharply reduced. Following consideration of several possible variables, Inkley concludes: "The smallest standard deviation from the line of regression occurred when plasma volume was plotted against weight or surface area. It appears from this study, that these two denominators offer the best method for evaluating or predicting plasma volumes."

Clinical diagnosis is a process of discriminating between the normal and the pathologic on the basis of a greater or smaller number of tests. Each test is associated with a cost in terms of time and money. The additional information resulting from employing an additional measure must be balanced against the cost of securing the measure. Both weight and surface area have been suggested as valuable measures for estimating normal plasma volume. The question here posed is whether both of these measures should be considered or whether either one alone provides all of the information of value. Obviously, the cost involved is nominal; so the question is essentially whether or not anything of value is to be gained by considering both measurements.

Body weight, surface area, and RISA estimates of plasma volume are presented for 48 individuals in the study of Inkley and associates. The correlation between weight and plasma volume is 0.6856; between surface area and plasma volume the correlation is 0.7337; and between weight and surface area the correlation is 0.9533. Plasma volume is observed to have somewhat greater regression upon surface area than upon body weight alone. The correlation of 0.7337 indicates that the expected variance in plasma volume estimates is reduced by 54 per cent by considering the regression upon surface area. The question of importance is whether anything *additional* can be gained by considering the regression of plasma volume upon body weight. The answer is no. After variance associated with surface area has been extracted, consideration of body weight results in no additional reduction in variance of plasma volume estimates.

This result is not surprising since surface area is a complex variable derived in part from body weight. The next question is whether the consideration of surface area results in significant additional information which could not be obtained from weight alone. The correlation of 0.6856 indicates that the expected variance in plasma volume is reduced by 47 per cent by considering the regression of that variable upon body weight. When surface area is included in the predictive equation, an additional 7 per cent of the variance in plasma volume is accounted for. This contribution tested as an F with 1 and 45 degrees of freedom is statistically significant at the 0.02 level. Therefore, it is concluded that when the information is available surface area should be employed as the best single predictor of normal plasma volume.

SUMMARY

1. When employed by a single group of investigators, Evans blue dye and RISA techniques for determining human plasma volume were found not to measure the same aspects or characteristics of experimental subjects. Estimates of plasma volume were consistently larger for some individuals when determinations were based upon one technique, while estimates based upon the other technique were consistently larger for other individuals.

2. Techniques of estimating plasma volume are significantly influenced by the technicians employing them. Evans blue dye and RISA are not two standard techniques but instead represent two families of techniques, members of which must be identified according to the investigators using them.

3. *Either* body weight or surface area is significantly predictive of plasma volume; however, surface area is a significantly better predictor than body weight. Nothing is gained from considering *both* surface area and body weight together.

REFERENCES

1. Zipf, R. E., Webber, J. M., and Grove, G. R.: A Comparison of Routine Plasma Volume Determination Methods Using Radioiodinated Human Serum Albumin and Evans Blue Dye (T-1824), J. Lab. & Clin. Med. **45**: 800-805, 1955.
2. Inkley, S. R., Brooks, L., and Krieger, H.: A Study of Methods for the Prediction of Plasma Volume, J. Lab. & Clin. Med. **45**: 841-850, 1955.

THE VALUE OF SIMULTANEOUS URINE BILIRUBIN AND SERUM ALKALINE PHOSPHATASE ANALYSES IN THE DIAGNOSIS OF INTRAHEPATIC METASTASES OF CARCINOMA

EDWARD J. FITZSIMONS, PH.D., HERBERT M. SOMMERS, M.D., AND THOMAS C. LAIPPLY, M.D.
CHICAGO, ILL.

FOR more than 20 years the finding of elevated serum alkaline phosphatase associated with a normal serum bilirubin level in patients with a malignant epithelial tumor, has served as an indication of metastases to the liver.[1-15] However, patients with diseases other than intrahepatic metastases, e.g., amebic abscess, viral hepatitis, cirrhosis, acute and chronic cholecystitis, cholangitis, sarcoidosis, tuberculosis, amyloidosis, Hodgkin's disease, lupus erythematosus, and cardiac failure with hepatic involvement, have been found to have an elevated serum alkaline phosphatase level in the absence of hyperbilirubinemia.[3-5, 13, 16, 17]

Shay and Siplet[13] attempted to enhance the specificity for detecting hepatic metastases by extending the necessary criteria. They recommended the use of the Bromsulphalein dye analysis in patients with elevated serum alkaline phosphatase levels and normal serum bilirubin for the detection of secondary tumor of the liver. However, increased dye retention was not limited to the presence of hepatic metastases. Increased Bromsulphalein retention, increased serum alkaline phosphatase, and normal serum bilirubin were found in cases of hepatic granulomas, choledocholithiasis, hepatitis without jaundice, and cirrhosis, as well as in patients with metastases. If, in addition, serum flocculation tests were normal, hepatic metastases were considered more likely to be present.

Assuming that a portion of the increase in serum alkaline phosphatase level in patients with hepatic metastases is due to intrahepatic obstruction, renal clearance of circulating "direct reacting" bilirubin may be at a sufficiently rapid rate to prevent the serum bilirubin level from becoming elevated. Since an increased excretion would then precede any actual elevation of serum level, the measurement of bilirubin excretion rate may serve as a sensitive indicator of intrahepatic obstruction. The present study was undertaken to determine whether this measurement contributed to further the specificity or reliability of predicting the presence of hepatic metastases in patients with increased alkaline phosphatase and normal serum bilirubin levels.

From the Department of Pathology and Biochemistry, Chicago Wesley Memorial Hospital, and Northwestern University Medical School, Chicago, Ill.

This investigation was made possible by a grant from The Service League of Chicago Wesley Memorial Hospital.

Received for publication June 4, 1958.

TABLE I. RESULTS OF URINE BILIRUBIN ANALYSES ON 61 CONSECUTIVE PATIENTS WITH AN ELEVATED SERUM ALKALINE PHOSPHATASE AND A NORMAL SERUM BILIRUBIN

PATIENTS WITH CARCINOMA						PATIENTS WITHOUT CARCINOMA			
CASE NUMBER	PRIMARY SITE	ALK. PHOS.	URINE BILIRUBIN	HEPATIC MET.	BONE* INVOLVEMENT	CASE NUMBER	DIAGNOSIS	ALK. PHOS.	URINE BILIRUBIN
		A. Urine Bilirubin Greater Than 0.25 mg. per 2 Hr.							
1	Bile duct	15.8	0.25	+	O	23	Lymphosarcoma	11.0	0.37
2-3	Breast	11.1	0.27	+	L	24	Hyperthyroidism	14.0	0.31
		10.2	0.26	+	O	25	Gastric ulcer	8.1	0.38
4	Gall bladder	8.9	0.47	+	O	26	Idiopathic sprue	63.1	0.26
5-8	Large intestine	39.3	1.12	+	L	27	Schistosoma mansoni hepatitis	55.0	0.51
		12.9	0.31	+	O				
		10.9	0.40	+	O	28	Viral hepatitis	20.0	0.29
		9.2	0.42	+	O	29	Fatty metamorphosis and bile pigmentation of liver	7.9	0.63
9	Liver	13.3	0.57	+	O				
10-11	Lung	8.1	0.81	+	L				
		31.2	1.02	+	O				
12-14	Ovary	7.4	0.32	+	O				
		6.5	0.25	+	O				
		18.6	0.25	+	O				
15	Prostate	10.8	0.92	+	B				
16-20	Not established	35.6	0.63	+	O				
		22.1	0.31	+	O				
		19.3	0.28	+	O				
		17.4	0.56	+	O				
		17.2	0.26	+	O				
21	Endocervix	12.5	0.47	–	L				
22	Not established	9.6	0.33	–	O				
		B. Urine Bilirubin Less Than 0.25 mg. per 2 Hr.							
30-31	Breast	29.3	0.17	+	O	38	Lymphosarcoma	15.3	0.08
		11.5	0.09	–	L	39-40	Lymphatic leukemia	7.7	0.00
32-34	Large intestine	20.0	0.21	+	O			11.3	0.05
		7.7	0.05	+	O	41-43	Chronic cholecystitis with cholelithiasis	7.1	0.09
		10.0	0.13	+	O			27.0	0.15
35-36	Prostate	20.9	0.13	–	B			8.6	0.16
		15.0	0.10	–	B	44-45	Acute cholecystitis with cholelithiasis	9.9	0.04
37	Stomach	6.0	0.24	–	O			11.4	0.12
						46-49	Hepatitis	9.5	0.07
								9.6	0.10
								11.5	0.10
								9.1	0.24
						50	Cholangitis	12.7	0.23
						51	Cirrhosis	12.1	0.03
						52-57	Heart failure	7.7	0.01
								16.5	0.06
								14.3	0.14
								17.0	0.15
								12.3	0.20
								8.4	0.21
						58	Hyperthyroidism	12.1	0.15
						59-60	Sarcoidosis	31.5	0.15
								28.0	0.20
						61	Not established	14.5	0.00

*Key: O = no involvement; L = osteolytic metastases; B = osteoblastic metastases.

METHODS

Serum alkaline phosphatase was determined by the method of Bodansky[18] as revised by Gomori.[19] The normal range is 1.4 to 4.0 units per 100 ml. of serum. The standard deviation for this method in our laboratory is 0.8 unit. Values greater than 6.0 units were considered significantly elevated. Total serum bilirubin was measured by the method of Malloy and Evelyn.[20] The normal range is 0.2 to 1.0 mg. per 100 ml. of serum. The standard deviation is 0.1 mg. Values greater than 1.2 mg. per 100 ml. of serum were considered

significantly elevated. Urine bilirubin was determined on 2-hour specimens by the method of Golden and Snavely.[21] A normal range of 0.0 to 0.2 mg. per 2 hours and a standard deviation of 0.06 mg. were established. Excretion rates greater than 0.25 mg. in a 2-hour period were considered significantly elevated.

Bilirubin excretion rates were determined for 61 consecutive patients without primary bone disease who had been found to have elevated serum alkaline phosphatase and normal serum bilirubin levels. Twenty-nine of these patients had a urine bilirubin excretion rate greater than 0.25 mg. per 2 hours (Table I). Of the 29, 20 (69 per cent) had clinical or anatomic evidence of secondary carcinoma in the liver. The remaining 9 included 2 patients with carcinoma, but no evidence of liver metastases, and 7 patients without carcinoma. The rate of renal bilirubin excretion was less than 0.25 mg. per 2 hours in 32 patients. Four of these patients (12.5 per cent) had hepatic metastases (Table I). Four others had carcinoma, but no evidence of hepatic metastases, and the remaining 24 patients were free of carcinoma.

DISCUSSION

Bilirubin, a product of hemoglobin metabolism, is normally excreted by the liver conjugated with glucuronic acid as a water-soluble glucuronide.[22-25] Obstruction of the biliary canaliculi causes the excretion of bilirubin glucuronide to be impaired and results in the excretion of this bilirubin fraction by the kidneys. As the degree of obstruction increases, the serum levels of one minute "direct reacting" bilirubin and total bilirubin rise. In most cases of biliary obstruction, e.g., those caused by calculi or inflammation, the interval between the time of increased urinary bilirubin clearance and increased serum level is usually only a few days. However, a slowly developing, possibly multifocal, obstruction due to metastases of carcinoma might be expected to result in the lengthening of this interval and permit detection of the metastatic process by means of the proposed analyses. The occurrence of an increased "direct reacting" serum bilirubin level with a normal total bilirubin has been reported in some patients with hepatic metastases.[26] However, less than one-fourth of the 24 patients reported here with hepatic metastases, an alkaline phosphatase above 6.0 Bodansky units, and a normal serum total bilirubin level had a serum level of one minute "direct reacting" bilirubin above 0.2 mg. per 100 ml. The data published by Shay an Siplet[13] is consistent with this.

The value of the serum alkaline phosphatase determination in the recognition of hepatic metastases from carcinoma has been well established. Two hundred and twenty-five of 292 patients (77 per cent) with metastatic carcinoma of the liver compiled from the literature had an elevated serum alkaline phosphatase level and a normal serum bilirubin level.[1-15] Preliminary to the present investigation, 14 of 24 patients (57 per cent) proved at autopsy to have hepatic metastases from carcinoma were found to have had a serum alkaline phosphatase level above 6.0 Bodansky units and a normal serum bilirubin level. The finding of an elevated serum alkaline phosphatase and a normal serum bilirubin level in patients known to have carcinoma was especially significant, 14 of 15 such patients (93 per cent) demonstrating hepatic metastases at autopsy.

The use of two criteria, elevated serum alkaline phosphatase and normal serum bilirubin levels, as a means of screening all patients (i.e., patients with

or without known carcinoma) for hepatic metastases of carcinoma is not sufficiently reliable. Gibbons[15] reported that 75 of 305 patients (25 per cent) with an elevated serum alkaline phosphatase and a normal serum bilirubin level had proved or probable hepatic metastases. Data obtained during the present study support this report. Twenty-four per cent of the patients (41 of 173) with the above two laboratory findings had hepatic metastases. In addition, however, when the rate of urine bilirubin excretion was determined (Table I) and found to be elevated, 69 per cent (20 of 29 patients) had metastatic carcinoma in the liver. Only 4 of 32 patients with a normal rate of excretion had hepatic metastases.

The alkaline phosphatase determination is not a reliable means of detecting the presence of hepatic metastases in patients having a primary carcinoma that frequently metastasizes to bone, particularly mammary and prostatic carcinomas. In carcinoma of the breast the metastases are predominantly osteolytic, whereas in carcinoma of the prostate the metastases are predominantly osteoblastic. According to Woodward,[27] in patients with osteolytic metastases the serum alkaline phosphatase level may be normal or only slightly increased, "to 6-7 Bodansky units," whereas osteoblastic metastases result in a significant increase in the serum alkaline phosphatase level. It has been reported that the finding of an increased serum glutamic oxalacetic transaminase level in patients with metastatic carcinoma to bone is indicative of the presence of concomitant liver metastases.[28] However, an increase in the concentration of this enzyme in serum is nonspecific and only indicates focal tissue necrosis.

The inclusion of urine bilirubin determinations aids in the recognition of hepatic metastases in patients with carcinoma having bone involvement. Five patients with predominantly osteolytic metastases are included in Table I. Three of these 5 had secondary tumor in the liver and were found to have an increased rate of urine bilirubin excretion. One of the two without evidence of hepatic metastases also had an increased rate of bilirubin excretion, a discrepancy which lacks explanation. Of 3 patients with predominantly osteoblastic metastases, 1 had hepatic metastases and an increased rate of urine bilirubin excretion. A normal rate of excretion was found in the other 2 (Table I). Although the number of patients studied is small, the results are encouraging.

SUMMARY

The determination of the rate of urinary bilirubin excretion in patients with increased serum alkaline phosphatase and normal serum bilirubin levels offers a more specific means of predicting the presence of metastatic carcinoma in the liver than do the latter two findings alone. Sixty-nine per cent of patients with a urine bilirubin excretion rate greater than 0.25 mg. per 2 hours, a serum alkaline phosphatase above 6.0 Bodansky units, and a normal serum bilirubin level were found at autopsy or operation to have hepatic metastases of carcinoma. The determination of the rate of urinary bilirubin excretion may also be of value in recognizing liver metastases in patients who have carcinoma metastasizing to bone.

REFERENCES

1. Gutman, A. B., Swenson, P. C., and Parsons, W. B.: The Differential Diagnosis of Hyperparathyroidism, J. A. M. A. **103**: 87-94, 1934.
2. Gutman, A. B., Tyson, T. L., and Gutman, E. B.: Serum Calcium, Inorganic Phosphorus and Phosphatase Activity in Hyperparathyroidism, Paget's Disease, Multiple Myeloma and Neoplastic Disease of the Bones, Arch. Int. Med. **57**: 379-413, 1936.
3. Flood, C. A., Gutman, E. B., and Gutman, A. B.: Phosphatase Activity, Inorganic Phosphorus and Calcium of Serum in Disease of Liver and Biliary Tract, Arch. Int. Med. **59**: 981-999, 1937.
4. Meranze, D. R., Meranze, T., and Rothman, M. M.: Serum Phosphatase as an Aid in the Diagnosis of Metastasis of Cancer to the Liver, Pennsylvania M. J. **41**: 1160-1164, 1938.
5. Gutman, A. B., Olson, K. B., Gutman, E. B., and Flood, C. A.: Effect of Disease of the Liver and Biliary Tract Upon the Phosphatase Activity of the Serum, J. Clin. Invest. **19**: 129-152, 1940.
6. Klotz, S. D.: An Analysis of Liver Function by a Simplified Combination of Laboratory Tests, Bull. New York M. Coll., Flower & Fifth Ave. Hosps. **6**: 1-23, 1943.
7. Bullard, R. W., Jr.: Alkaline Phosphatase and Metastatic Liver Disease, Surgery **19**: 379-382, 1946.
8. Ariel, I. M., and Shahon, D. B.: Hepatic Dysfunction in Candidates for Abdominal Surgery, Especially Patients With Cancer, Cancer **3**: 608-623, 1950.
9. Burke, J. O.: Serum Alkaline Phosphatase in Liver Disease: A Concept of Its Significance, Gastroenterology **16**: 660-668, 1950.
10. Cabello, J., Silva, S., and Icekson, I.: Estudio de las fosfatasas alcalinas del suero en las neoplasias y su valor diagnóstico frente a las metástasis hepáticas, Rev. méd. Chile **79**: 157-161, 1951.
11. Mendelsohn, M. L., and Bodansky, O.: The Value of Liver-Function Tests in the Diagnosis of Intrahepatic Metastases in the Nonicteric Cancer Patient, Cancer **5**: 1-8, 1952.
12. Church, D., and Blackburn, C. R.: An Evaluation of Liver Function Tests, Including Filter Paper Electrophoresis, Australasian Ann. Med. **3**: 279-288, 1954.
13. Shay, H., and Siplet, H.: The Value of Serum Alkaline Phosphatase and Bromsulphalein Test in the Diagnosis of Metastatic Cancer of the Liver, J. LAB. & CLIN. MED. **43**: 741-751, 1954.
14. Simons, R. L.: Hepatic Tests in Metastatic Carcinoma of the Liver, Am. J. M. Sc. **228**: 312-316, 1954.
15. Gibbons, T. B.: Hyperphosphatasemia in Patients Without Jaundice With Hepatobiliary Disease, J. A. M. A. **164**: 22-28, 1957.
16. Ross, R. S., Iber, F. L., and Harvey, A. M.: The Serum Alkaline Phosphatase in Chronic Infiltrative Disease of the Liver, Am. J. Med. **21**: 850-856, 1956.
17. Rapoport, S.: Increased Serum Phosphatase and "Hyperprothrombinemia" in Infectious Hepatitis of Children, Proc. Soc. Exper. Biol. & Med. **62**: 203-207, 1946.
18. Bodansky, A.: Phosphatase Studies. II. Determination of Serum Phosphatase. Factors Influencing the Accuracy of the Determination, J. Biol. Chem. **101**: 93-104, 1933.
19. Gomori, G.: A Modification of Colorimetric Phosphorous Determination for Use With Photoelectric Colorimeter, J. LAB. & CLIN. MED. **27**: 955-960, 1942.
20. Malloy, H. T., and Evelyn, K. A.: The Determination of Bilirubin With the Photoelectric Colorimeter, J. Biol. Chem. **119**: 481-490, 1937.
21. Golden, W. R. C., and Snavely, J. G.: A Method for the Quantitative Determination of Bilirubin in Urine, J. LAB. & CLIN. MED. **33**: 890-903, 1948.
22. Schmid, R.: Direct-Reacting Bilirubin, Bilirubin Glucuronide, in Serum, Bile and Urine, Science **124**: 76-77, 1956.
23. Talafont, E.: Properties and Composition of the Bile Pigment Giving a Direct Diazo Reaction, Nature London **2**: 312, 1956.
24. Billing, B. H., and Lathe, G. H.: The Excretion of Bilirubin as an Ester Glucuronide Giving the Direct van den Bergh Reaction, Biochem. J. **63**: 6 (Proceedings), 1956.
25. Arias, I. M., and London, I. M.: Bilirubin Glucuronide Formation in Vitro; Demonstration of a Defect in Gilbert's Disease, Science **126**: 563-564, 1957.
26. Watson, C. J.: The Importance of the Fractional Serum Bilirubin Determination in Clinical Medicine, Ann. Int. Med. **45**: 351-368, 1956.
27. Woodward, H. Q.: Role of the Chemical Laboratory in Diagnosis of Neoplastic Disease of the Bone, Arch. Surg. **47**: 368-383, 1943.
28. Wroblewski, F., and LaDue, J. S.: Serum Glutamic-Oxalacetic-Transaminase Activity as an Index of Liver-Cell Injury From Cancer, Cancer **8**: 1155-1163, 1955.

A STUDY OF LIPIDS IN THE CEREBROSPINAL FLUID

IV. The Determination of Free and Total Cholesterol

Wallace W. Tourtellotte, Ph.D., M.D., Beverley A. Skrentny, B.S., and Russell N. DeJong, M.D.
Ann Arbor, Mich.

In A recent review of the literature on cholesterol in the cerebrospinal fluid (CSF),[1] it was deduced that the most important reason for the great differences in the cholesterol values in the spinal fluid reported by various investigators was the technologic difficulty in determining microquantities of cholesterol. In spite of this it was possible to establish from the most recent studies that there existed a minute amount of total cholesterol in normal spinal fluid which fell within a rather narrow range, 0.1 to 0.5 mg. per 100 ml. It had not been established that there was free cholesterol in normal spinal fluid.

The purpose of the present investigation was to devise an accurate method of analysis for free and total cholesterol on small quantities of spinal fluid. The most sensitive cholesterol procedure is the fluorometric method of Albers and Lowry.[2] The application of this procedure to the determination of cholesterol in 0.25 ml. of normal spinal fluid will be presented. From the literature review we found that the reliable Schoenheimer and Sperry procedure recently revised[3] had never been applied to spinal fluid. Certain modifications have made it possible to precipitate quantitatively free cholesterol as cholesterol digitonide from a lipid extract obtained from 1.75 ml. of normal CSF. The digitonide was purified and the cholesterol determined colorimetrically in 60 μl final volume.

MATERIALS AND METHODS

Reagents.—

1. Trichloroacetic acid (5 and 50 per cent): Trichloroacetic acid (TCA), analytical grade, was vacuum distilled and diluted with glass double-distilled water.[4]

2. Redistilled absolute ethyl alcohol: Commercial grade absolute ethyl alcohol was distilled through helices in a 800 mm. vacuum jacketed distilling column and the fraction boiling at 78° C. was taken.

3. Potassium acetate in absolute ethyl alcohol: Potassium acetate (0.2 N) is dissolved in redistilled absolute ethyl alcohol.

4. Redistilled 1,1,2-trichloroethane (TCE): The solvent obtained from Distillation Products Industries (No. T851) was distilled and the fraction boiling at 112 to 113° C. was taken. It was purified by washing 3 times with sulfuric acid (100 ml. of sulfuric acid per 1 L. of TCE) and 3 times with glass double-distilled water (250 ml. per 1 L. of TCE). It was dried by storing over anhydrous sodium sulfate.

From the Department of Neurology, University Hospital, and the Neurology Research Laboratory, Kresge Medical Research Building, University of Michigan Medical School, Ann Arbor, Mich.

This study was supported by grants from the National Multiple Sclerosis Society.
Received for publication Nov. 11, 1958.

5. Redistilled acetic anhydride: Acetic anhydride, analytical grade, was distilled and the fraction boiling at 137 to 138.5° C. was taken.

6. Sulfuric acid: Concentrated sulfuric acid, reagent grade, was purified by heating over a low flame in a hood with 5 per cent by volume of 72 per cent perchloric acid until the solution became colorless and fuming subsides (2 to 3 working days).

7. Diethyl ether, analytical grade: The ether was checked for peroxides regularly by shaking 10 ml. with 1 ml. of 10 per cent cadmium potassium iodide solution. If no color appeared after one hour in the dark, the ether was usable. It should be stored at 4° C.

8. Redistilled acetone: Acetone, commercial grade, was distilled and the fraction boiling at 56 to 58° C. was taken.

9. Digitonin solution: A 0.5 per cent digitonin (Hoffman-La Roche) solution was made in 50 per cent ethyl alcohol at 60° C.[3]

10. Acetone-absolute ethyl alcohol (1:1): The redistilled solvents were used.

11. Glacial acetic acid, analytical grade.

12. Acetic acid solution: Dilute 12 ml. of glacial acetic acid to 100 ml. with water.

13. Total cholesterol standards: Cholesterol (Eastman Kodak Company) was crystallized two times from redistilled acetone and dried in vacuo. Standard cholesterol solutions of 25, 50, and 100 μg per milliliter were prepared in glacial acetic acid.

14. Free cholesterol standards: Recrystallized cholesterol was used to make the following standard solutions: 250, 500, and 1,000 μg per milliliter in redistilled ethanol.

Apparatus.—

1. Reaction tubes (12 by 75 mm.): Pyrex culture tubes and 7 by 70 mm. bacteriologic tubes were pointed.

2. Fluorometer tubes: Pyrex tubes 10 by 75 mm. were selected by determining the fluorescence of 0.01 mg. per milliliter. Rhodamine B solution (aqueous solution). The tubes were etched at the top to indicate where the tube should be placed. The fluorometer tubes must be handled with care to prevent scratching and carefully cleaned before use.[2]

3. Parafilm (Marathon Corporation, Menasha, Wis.) was used for sealing tubes.

4. A Beckman DU spectrophotometer was adapted to read a volume of 50 μl and 1 cm. light path.*

5. A Farrand fluorometer was used for measuring the fluorescence. The λ 546 mμ mercury line isolated with Corning filters, Nos. 4010 and 5120, was used for the exciting source, and the secondary filter was the Corning No. 2424, a cutoff filter with 37 per cent transmittance at about λ 590 mμ.

Analytic Procedure.—The precipitation and washing steps were carried out in an ice water bath. The remaining steps were performed at room temperature. Lang-Levy pipettes were used for all transfers below 1 ml. and recalibrated volumetric pipettes for volumes over 1 ml. All mixings were accomplished by means of an off-center rotating cone (mixer) or "buzzer."[5] The tubes were not mixed so vigorously that protein precipitate was thrown up along the sides of the tubes out of reach of the extracting reagents. A centrifuge evaporator[2] was used to remove solvents.

Step 1. Precipitation and washing: This method was previously described.[6] Spinal fluid was precipitated with TCA (final concentration 10 per cent); the precipitate was washed 2 times with 5 per cent TCA.

Step 2. Lipid extraction: Extraction was done as previously described.[6] The protein precipitate was extracted with alcoholic 0.2 N potassium acetate, absolute ethanol, and hot absolute ethanol.

Step 3. Determination of total cholesterol: A 75 μl aliquot was taken from the 1 ml. volumetric flask containing the lipid extract and transferred to a fluorometer tube. The

*All equipment necessary to adapt the Beckman spectrophotometer to microanalysis was obtained from the Pyrocell Manufacturing Company, 207 East 84th Street, New York 28, N. Y.

extract was dried completely in about 40 minutes in the centrifuge evaporator. Twenty microliters of glacial acetic acid was added to the dried contents of the tube and buzzed. One milliliter of a fresh solution of trichloroethane and acetic anhydride in a 5:1 ratio by volume was added and buzzed. The tube was mixed 2 or 3 times over a 20-minute period to allow for complete solution of the sample and complete reaction between the acetic anhydride and any water present. Thirty-seven microliters of sulfuric acid was added and buzzed promptly and vigorously. The tube was covered with aluminum foil and after 2 hours the fluorescence was determined in the Farrand fluorometer.

Step 4. Preparation of blanks and total cholesterol standards: Seventy-five microliters of a 0.02 N potassium acetate in absolute ethanol solution was added to 9 fluorometer tubes. The blanks were dried with the lipid extracts. Twenty microliters of glacial acetic acid were added to the first 3 tubes. Twenty microliters of cholesterol standards with concentrations of 25, 50, and 100 mg. per milliliter of glacial acetic acid were added in duplicate to the remaining 6 tubes. The color was developed as for the lipid extract.

Step 5. Determination of free cholesterol:

A. Precipitation and Washing: A 575 μl aliquot was taken from the 1 ml. volumetric flask containing the lipid extract and transferred to a 7 by 70 mm. reaction tube. The extract was dried by means of a centrifuge-evaporator, the time required being about one hour. The residue was dissolved in 60 μl of acetone-ethanol (1:1). Five microliters of 12 per cent acetic acid solution was added, and the tube was buzzed again. About 30 μl of alcoholic digitonin solution was then added rather slowly, and the tube buzzed continually until the precipitate began to appear (sometimes two minutes with the lowest cholesterol concentrations). The tube was sealed with Parafilm and placed in a 38° C. water bath for 3 hours. The tube was centrifuged at 1,600 RCF for 15 minutes. The supernatant fluid was aspirated and discarded. The tube was washed with 100 μl of freshly made acetone-ethyl ether (1:2); the sides of the tube were carefully rinsed. The tube was buzzed, capped with Parafilm, and centrifuged at 4° C., 2,200 RCF for 15 minutes. The supernatant fluid was again aspirated and discarded. The washing step was repeated twice more with ethyl ether. The washed digitonide may be left at this stage for several days. If the color is developed immediately, the tube should be placed in a 38° C. water bath for 3 or 4 minutes to remove the last traces of ethyl ether.

B. Color Development: The tube was placed in a 110° C. sand bath for 30 minutes. The sand bath was removed from the oven, and while the tube was still in the sand, 20 μl of glacial acetic acid was added. It was then removed and buzzed. (The acid may be added to 3 or 4 tubes at a time before removing and buzzing.) Acetic anhydride-sulfuric acid (20:1) was made up fresh by adding 0.1 ml. of sulfuric acid to 2 ml. of cold acetic anhydride and kept in an ice bath. At a rate of one tube per minute, 40 μl of acetic anhydride-sulfuric acid solution was added and the tube was buzzed. It was then capped with Parafilm and placed in a 25° C. dark cabinet water bath. The reaction was read in a Beckman spectrophotometer (625 mμ) between 27 and 32 minutes after the addition of the acetic anhydride-sulfuric acid solution.

Step 6. Preparation of blanks and free cholesterol standards: Five hundred and seventy-five microliters of a 0.02 N potassium acetate in ethanol solution was added to 9 pointed 7 by 70 mm. reaction tubes. Five microliters of cholesterol standards in ethanol with concentrations of 250, 500, and 1,000 μg per milliliter were added in duplicate to the last 6 tubes. The ethanol was removed by means of the centrifuge-evaporator. The residue was dissolved in 60 μl of acetone-ethanol (1:1) and treated as the lipid extract described under Step 5, A.

Comments on the Analytic Procedure.—

Step 1: Varying the final concentration of the TCA from 10 to 16 per cent at the time the lipids were coprecipitated with the protein appeared to cause little variation in results. The lower final concentration was used. There was no significant difference in the

final results whether one, two, or no washes of 5 per cent TCA were used. However, there was a problem with insoluble matter in subsequent procedures when no wash was used, and possibly when only one wash was employed. Two 500 μl washes appeared sufficient.

Blank readings or the standard curves were not affected by 5, 10, or 20 μl of 5 per cent TCA or as much as 200 μl of 0.2 N potassium acetate in ethanol.

Step 2: There was evidence that the first extraction (100 μl of 0.2 N potassium acetate in ethanol) removed most of the cholesterol. To be cautious and hence to avoid contamination of the subsequent samples, it is recommended that after the transfer of the first extract, the pipette be filled once with ethanol and the rinse transferred to the 1 ml. volumetric tube.

Step 3: It was found that the dried lipid residue for the total cholesterol determination was more soluble in glacial acetic acid than trichloroethane-acetic anhydride (5:1) which was recommended by Albers and Lowry.[2] This additional reagent did not interfere with the fluorescence.

In Fig. 1 the rate of fluorescence development was plotted for a specimen of cerebrospinal fluid (CSF) and for 0.8 μg of cholesterol standard. It can be seen that the cholesterol standard reached a peak fluorescence between 1 and 2 hours, while the CSF appeared to peak between 2 and 3 hours. It was decided from experiments of this type that 2 hours after the addition of sulfuric acid was an appropriate time for determination of fluorescence.

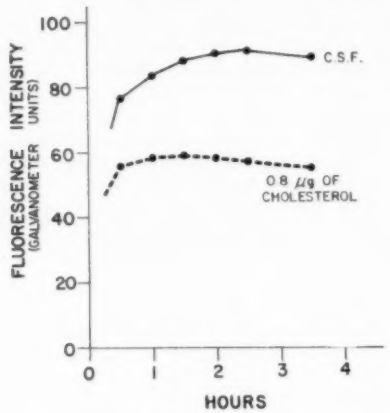


Fig. 1.—Rate of fluorescence development.

Step 4: Fig. 2 illustrates a typical standard curve for total cholesterol. The highest standard represented is 2.00 μg per sample or aliquot. It has been demonstrated that the results were linear to 6.00 μg and quenching was very definite at 12 μg of cholesterol. The equation of the line obtained by the analysis of least squares[7] was $Y = 0.4 + 35.04\ \overline{X}$, where Y is the reading in galvanometer units, 0.4 is the intercept, 35.04 is the slope, and $\overline{X}$ is the microgram of cholesterol in the sample. Inspection of the graph and the equation indicate that the intercept could be considered zero.

Calculations: From the equation of the line of the cholesterol standard curve, the micrograms of cholesterol were calculated for the sample. If 75 μl of the 1 ml. lipid extract of 3.0 ml. of CSF was used, then 444 times the micrograms found in the sample was equal to the micrograms of total cholesterol per 100 ml. of CSF.

Step 5: A. To determine the effective time for precipitation of cholesterol digitonide the following experiment was done. After 30 μl of digitonin solution was added to known cholesterol standards and the tube was buzzed continually until the precipitate began to

appear, one set of the samples was allowed to stand 18 hours at room temperature, while another set was placed in a 38° C. water bath for 3 hours. Triplicates of 3 concentrations of cholesterol were run in each set. The 18-hour period yielded an average 3 per cent increase in recovery. Since standards were carried through the precipitation and the difference was small between 3 and 18 hours, it was decided that the 3-hour incubation was sufficient.

Experiments were performed to determine the efficiency of the free cholesterol precipitation and the washing of the cholesterol digitonide formed. Three concentrations (triplicates) of cholesterol standards dissolved in absolute ethanol (1, 2, and 4 μg per sample) were carried through the digitonin precipitation; the washes and the color development were as described in Step 5, A and B. These results were compared with those obtained when the same cholesterol standards were not reacted with digitonin and were not washed but the color was developed as described in Step 5, B. For all 3 concentrations tested there was only a 10 per cent loss of color when the cholesterol standards were precipitated and washed as described. In other experiments, samples containing 0.5 μg of cholesterol gave erratic results. It is recommended that for greatest accuracy the cholesterol standards should be carried through the digitonin step.

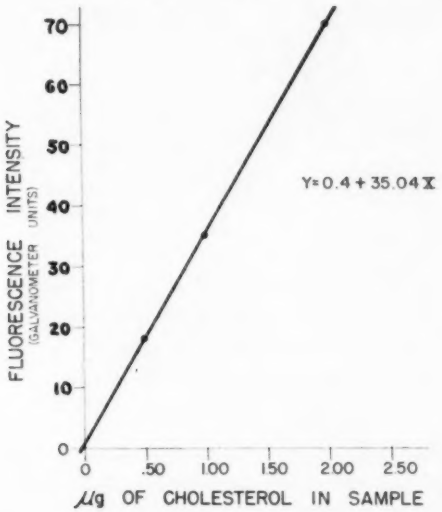


Fig. 2.—Total cholesterol standard curve.

B. An attempt was made to determine free cholesterol fluorometrically. Because digitonin fluoresced to the same extent as cholesterol when reacted with acetic anhydride and sulfuric acid it was necessary to remove all the excess digtonin from the precipitate. Ether was found to be inadequate because it precipitated the excess digitonin. Absolute ethanol, on the other hand, dissolved the cholesterol digitonide as well as the digitonin. The most effective wash was 25 per cent ethanol in water. After washing, the precipitate was dissolved in glacial acetic acid, and the fluorescence was developed as in the total cholesterol determination. Although the recovery of known samples of cholesterol ranged for the most part between 83 and 90 per cent, the results were more erratic and far less dependable than the colorimetric determination.

Step 6: Fig. 3 illustrates a typical standard curve for free cholesterol. The highest standard represented is about 4 μg per sample or aliquot. It has been demonstrated that the reaction is linear up to 20 μg per sample without any modification of the procedure.

Higher samples were not tested. The equation of the line obtained by the analysis of least squares[7] was $Y = -8 + 77.77\ \overline{X}$, where Y was the optical density times 1,000, where -8 was the intercept, where 77.77 was the slope, and where $\overline{X}$ was the microgram of cholesterol in the sample. No explanation could be found for the intercept.

Calculations: From the equation of the line of the free cholesterol standard curve, the micrograms of free cholesterol was calculated for the sample. If 575 μl of the 1 ml. lipid extract of 3.0 ml. of CSF was used, then 57.9 times the micrograms found in the sample was equal to the micrograms of free cholesterol per 100 ml. of CSF.

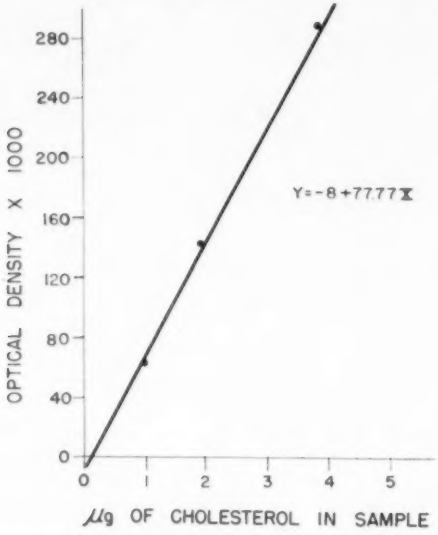


Fig. 3.—Free cholesterol standard curve.

RESULTS AND DISCUSSION

The coefficient of variation[7] for total and free cholesterol with the proposed procedures was 3.3 and 6.6 per cent, respectively (Table I). Therefore, if a number of extractions were carried out on a given CSF specimen, it could be expected from the proposed procedure that 67 per cent of all the determinations would fall within ±3.3 per cent of the mean for total cholesterol and ±6.6 per cent of the mean for free cholesterol. On the other hand, the coefficient of variation for total cholesterol was found to increase to ±7.1 and ±10.8 per cent if 2 or 1 ml. of CSF, respectively, was extracted. This tendency also was found for free cholesterol.

Experiments were designed to test the reproducibility of the proposed procedures on several different workdays. The CSF specimens were stored under sterile conditions at 4° C. between extractions. Analysis of a specimen of CSF yielded 348, 289, 316, and 334 μg of total cholesterol per 100 ml. of CSF, initially and after 5, 6, and 10 days, respectively. More elaborate testing along this line is shown in Table II. Inspection of the data indicates there was no difference between the results of the simultaneous extractions and between the results of the original extraction and extraction done 7 months later. It

would appear from these data that the concentrations of total and free cholesterol in CSF were not appreciably altered after storage of CSF under sterile conditions at 4° C. for as long as 7 months.

TABLE I. REPRODUCIBILITY EXPERIMENTS

DETERMINATION NO.	CHOLESTEROL μg/100 ML. OF CSF	
	TOTAL	FREE
1	586	195
2	593	200
3	552	217
4	620	237
5	601	225
6	600	190
7	607	224
8	606	221
9	605	215
10	621	217
11	621	220
Mean	601	215
Standard deviation	19.8	14.1
Coefficient variation	3.3%	6.6%

TABLE II. REPRODUCIBILITY OF RESULTS OVER A PERIOD OF TIME

CSF REFERENCE	CHOLESTEROL DATA INITIALLY		CHOLESTEROL DATA 7 MONTHS LATER	
	TOTAL	FREE	TOTAL	FREE
236	325 (323)	115 (108)	342 (297)	154 (162)
259	827	314	754 (722)	348 (328)
W1	415	141	425	140
W2	416	144	268*	128
252	414 (395)	166 (166)	427 (393)	201 (161)

Parentheses indicate simultaneous extraction.

All results are the average of duplicate determinations and are expressed as micrograms per 100 ml. of CSF.

*This low value was double checked.

TABLE III. REPRODUCIBILITY OF THE PROPOSED PROCEDURE ON DIFFERENT AMOUNTS OF CSF

EXTRACT REFERENCE	μl OF CSF USED	
	100	200
First	348	296
First	313	308
First	345	313
Second	289	263
Third	316	340
Fourth	334	307

All experiments were carried out on 200 μl of 1.0 ml. of lipid extract equivalent to 1.0 or 2.0 ml. of CSF; hence, each determination was done on 100 or 200 μl of CSF, respectively. All total cholesterol values are expressed in micrograms per 100 ml. of CSF and are the average of duplicates.

The data in Table III illustrate the reproducibility of the proposed total cholesterol procedure on different amounts of CSF. With the coefficient of variation found, the data indicated that there was no significant difference between extraction of 1.0 or 2.0 ml. of CSF.

The Sperry-Webb[3] method for the extraction and measurement of total cholesterol from serum was compared to the proposed procedure. For the Sperry-Webb procedure, 0.2 ml. of serum was extracted in triplicate as they directed. Samples of the same serum were also diluted 1 to 500 in 0.9 per cent

sodium chloride (this dilution approximates the concentration of total cholesterol in CSF), and 3.0 ml. of the diluted serum was extracted and the fluorometric determinations were carried out as proposed for CSF. The data in Table IV illustrate on 5 different serum specimens that the proposed procedure was quantitative, 90.3 per cent of the Sperry-Webb procedure.

TABLE IV. COMPARISON OF THE PROPOSED TOTAL CHOLESTEROL PROCEDURE WITH THE METHOD OF SPERRY AND WEBB[3] ON SERUM

SERUM REFERENCE	SPERRY-WEBB METHOD	PRESENT METHOD
1	258	219
2	211	190
3	476	403
4	223	204
5	268	270

All values are expressed in milligrams per 100 ml. of CSF and are the average of triplicates.

The concentration of total cholesterol in CSF is several hundred times too low for analysis with the Sperry-Webb procedure. Therefore, 350 ml. of CSF was pooled and after aliquots were taken for total cholesterol analysis by the proposed fluorometric microprocedure, four 30 ml. and three 60 ml. samples of the pooled CSF were lyophilized. The dried material was extracted by the procedure of Brante.[8] The lipid extract was purified by Sperry-Brand's Procedure 2.[9] After purification the solution was evaporated to dryness at 38° C. in a nitrogen stream without transferring. The residue obtained was dissolved in acetone-absolute ethanol (1:1) and the Sperry-Webb total cholesterol procedure[3] was carried out. The total cholesterol values obtained were 264, 307, 282, and 285 μg per 100 ml. of CSF for the 30 ml. samples and 298, 263, and 265 μg per 100 ml. of CSF for the 60 ml. samples. The average was 281 μg per 100 ml. of CSF. The concentration obtained by the proposed procedure was 327 or 116 per cent of the concentration obtained by the Sperry-Webb procedure.

Since the difference between the two procedures on CSF as mentioned in the previous paragraph (116 per cent) could be related to the technologic difficulties in extracting micrograms of lipids (100 to 200 μg of cholesterol) from dried solids with large quantities of organic solvents, hence producing a net loss or incomplete extraction of cholesterol from lyophilized CSF, the following experiment was designed. The extraction procedure (Steps 1 through 6) was used to prepare the lipid extract. The Sperry-Webb total cholesterol procedure was then adapted to microanalysis in the following fashion. To 400 μl of the 1 ml. of lipid extract, 20 μl of 10 N potassium hydroxide was added, and the mixture was incubated at 38° C. for 60 minutes. Twenty microliters of 12 per cent acetic acid solution was sufficient to acidify the saponified lipid extract; 250 μl of the digitonin solution was added and incubated at 38° C. for 3 hours and then 18 hours more at room temperature. The cholesterol digitonide was washed as described by Sperry and Webb except 100 μl of solvent was used. The color was developed also as described[3] except 20 μl of glacial acetic acid and 40 μl of acetic anhydride-sulfuric acid mix were added; blanks and

standards were carried through the entire procedure and treated as the unknowns. The comparison of the proposed total cholesterol procedure with the micromethod of Sperry and Webb on 4 CSF specimens extracted as directed (Steps 1 through 6) is shown in Table V. It can be seen that the agreement was good for these two different procedures, 106.2 per cent recovery (assuming the Sperry-Webb results to be the true value).

TABLE V. COMPARISON OF THE PROPOSED TOTAL CHOLESTEROL PROCEDURE WITH THE MICROMETHOD OF SPERRY AND WEBB ON THE PROPOSED CSF LIPID EXTRACT

CSF REFERENCE	MICROMETHOD OF SPERRY AND WEBB	PRESENT METHOD
A	431	459
B	393	365
C	253	317
D	624	624

All values are expressed in milligrams per 100 ml. of CSF and are the average of duplicate determinations.

The possibility of hydrolyzing the esterified cholesterol to free cholesterol in the presence of the alkaline ethanol (20 μEq of potassium acetate in the 1 ml. lipid extract) to produce a falsely high free cholesterol value has been seriously considered. Several serum studies were carried out in which free cholesterol was determined according to the procedure described by Sperry and Webb.[3] The same sera were also diluted 1:500 in 0.9 per cent sodium chloride to obtain the expected cholesterol concentration found in CSF. The free cholesterol was then determined by the proposed procedure. The proposed micromethod yielded 3 per cent more cholesterol than the macromethod of Sperry and Webb. Furthermore, determinations have been done on lipid extracts stored at 4° C. as long as 5 weeks after preparation without any significant difference in the free cholesterol concentration between the initial and later determination.

Further support of the reliability of the proposed micromethods for determination of free and total cholesterol has been reported recently. Galloway and associates[10] adapted from the method of Sperry and Webb a microdetermination for free and total cholesterol in which only 0.04 ml. of blood serum was necessary. Also Carpenter and co-workers[11] estimated the total cholesterol in serum by the Albers and Lowry fluorometric technique in which only 0.02 ml. of serum was required.

SUMMARY

An analytic method has been presented which is capable of determining 1 μg of free cholosterol and 0.5 μg of total cholesterol in CSF (less than 2 ml. of normal CSF) with a coefficient of variation of about 3 and 7 per cent, respectively. It was based on the coprecipitation of proteins and lipids and the colorimetric determination of free cholesterol (cholesterol digitonide) and the fluorometric determination of total cholesterol on a suitable lipid extract. Several tests for the validity of the proposed procedure on CSF were presented.

REFERENCES

1. Tourtellotte, W. W., DeJong, R. N., and van Houten, W. H.: A Study of Lipids in the Cerebrospinal Fluid. I. The Historical Aspects, Univ. Michigan M. Bull. **24**: 66-96, 1958.
2. Albers, W., and Lowry, O. H.: Fluorometric Determination of 0.1 to 10 Micrograms of Cholesterol, Anal. Chem. **27**: 1829-1831, 1955.
3. Sperry, W. M., and Webb, M.: A Revision of the Schoenheimer-Sperry Method for Cholesterol Determination, J. Biol. Chem. **187**: 97-106, 1950.
4. Tourtellotte, W. W., Parker, J. A., Alving, R. E., and DeJong, R. N.: Determination of Total Protein in Cerebrospinal Fluid by an Ultramicro-Kjeldahl Nitrogen Procedure, Anal. Chem. **30**: 1563-1566, 1958.
5. Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M.-L., and Farr, A. L.: The Quantitative Histochemistry of Brain. I. Chemical Methods, J. Biol. Chem. **207**: 1-17, 1954.
6. Tourtellotte, W. W., Vander, A. J., Skrentny, B. A., and DeJong, R. N.: A Study of Lipids in the Cerebrospinal Fluid. II. The Determination of Total Lipids, J. Lab. & Clin. Med. **52**: 481-490, 1958.
7. Arkin, H., and Colton, R. R.: Statistical Methods, New York, 1956, Barnes & Noble, Inc.
8. Brante, G.: Studies on Lipids in the Nervous System With Special Reference to Quantitative Chemical Determination and Topical Distribution, Acta physiol. scandinav. **18** (Suppl. 63): 1-189, 1949.
9. Sperry, W. M., and Brand, F. C.: The Determination of Total Lipids in Blood Serum, J. Biol. Chem. **213**: 69-76, 1955.
10. Galloway, L. S., Nielson, P. W., Wilcox, E. B., and Lantz, E. M.: Micro Determination of Cholesterol by Use of 0.04 ml. of Blood Serum, Clin. Chem. **3**: 226-232, 1957.
11. Carpenter, K. J., Gotsis, A., and Hegsted, D. M.: Estimation of Total Cholesterol in Serum by a Micro Method, Clin. Chem. **3**: 233-238, 1957.

MUCOPROTEINS OF CEREBROSPINAL FLUID AND BLOOD IN NEUROLOGIC DISORDERS

A. Zlotnick, M.Sc., M.D., E. Weisenberg, Ph.D., and I. Chowers, M.D.
Jerusalem, Israel

THE mucoproteins were defined by Winzler and associates[1] as a carbohydrate-rich protein fraction which remains soluble after precipitation of the serum proteins with perchloric acid, and is precipitated by an addition of phosphotungstic acid. The clinical significance of this serum fraction has been reviewed by Greenspan,[2] Winzler,[3] and Stary.[4] Its level is increased in acute and chronic infections, myocardial infarction, metastatic neoplasms, and after experimental irradiation in animals; it is reduced in hepatic cirrhosis and lipoid nephrosis.

While the recent medical literature is rich in studies on the variations of the mucoproteins found in the blood in different diseases, only scarce information is given with regard to their presence in the cerebrospinal fluid.[5, 6] The following is an attempt to assess the clinical value of the mucoprotein determination in cerebrospinal fluid and blood in a variety of neurologic disorders.

MATERIAL AND METHODS

Forty-nine patients, admitted to the Department of Neurology for various neurologic disorders, served as subjects for this study. In each case the diagnosis was established by the usual neurologic criteria. Where a space-occupying lesion was suspected the patient was submitted to pneumoencephalographic and arteriographic examinations. In some of the cases a ventriculogram or an operation verified or disproved the diagnosis. The detailed diagnoses of the patients are given in Table I. As control material, blood from 12 healthy students and the cerebrospinal fluid of 8 patients who were free from any systemic disease known to affect the central nervous system, was used.

Spinal fluid was obtained by lumbar puncture, and blood was taken from the cubital vein. The total protein of the spinal fluid was determined by the method of Lowry and associates.[7] The mucoprotein fraction was determined within 24 hours after obtaining the material. In a few cases the cerebrospinal fluid was kept for 48 to 72 hours at a temperature of –20° C. before the performance of the test.

From the Departments of Nervous Diseases and Internal Medicine A, Rothschild Hadassah University Hospital and Hebrew University, Hadassah Medical School, and the Government Institute for Standardization and Control of Pharmaceuticals, Ministry of Health, Jerusalem, Israel.

Received for publication Dec. 5, 1958.

TABLE I. THE CEREBROSPINAL FLUID AND SERUM MUCOPROTEINS IN DIFFERENT NEUROLOGIC DISORDERS

NUMBER	PATIENT	AGE	DIAGNOSIS	SERUM MUCOPROTEIN (MG. %)	CEREBROSPINAL FLUID		REMARKS
					PROTEIN (MG. %)	MUCOPROTEIN (MG. %)	
A. Controls							
12	Students	24-32	Healthy	74.2 ± 10.4			
1	R. S.	35	Rheumatoid arthritis		—	6.4	
2	G. A.	14	Mental retardation		26	4.7	
3	B.	60	Diabetes		46	7.8	
4	P. L.	44	Neurasthenia		57	6.8	
5	S. J.	22	Neurasthenia		39	2.3	
6	L. J.	31	Hemoptysis		28	4.6	
7	P. S.		Schizophrenia		—	3.8	
8	N. E.		Schizophrenia		—	4.0	
Mean and standard deviation					39.2 ± 13	5.1 ± 1.8	
B. General Neurologic Disorders							
1	B. E.	36	Left hemiparkinson	118	30	7.0	
2	B. J.	38	Left hemiparkinson	119	29	6.8	
3	M. S.	42	Parkinson syndrome	84	22	8.6	
4	G. D.	49	Multiple sclerosis	99	61	8.4	
5	G. Sh.	48	Multiple sclerosis	99	46	6.3	
6	M. M.	36	Multiple sclerosis	90	92	5.9	
7	B. P.	21	Multiple sclerosis	82	49	5.6	
8	K. F.	45	Multiple sclerosis	100	45	7.7	
9	M. A.	56	Amyotrophic lateral sclerosis	76	56	6.4	
10	Sh. E.		Cerebrovascular accident				
			Urinary infection	198	50	6.0	
11	K. B.	58	Cortical blindness, hypertensive encephalopathy	88	100	4.2	
12	W. R.	72	Cerebrovascular accident	194	49	6.4	
13	P. J.	48	Thrombosis of int. carotid artery	146	—	8.2	
14	D. N.		Cortical atrophy	230	21	5.6	
15	B. T.	44	Hydrocephalus	99	29	5.9	
16	O. J.	64	Cortical atrophy	116	26	6.5	
17	G. L.	59	Cortical atrophy, hydrocephalus	—	47	6.2	
18	S. Ch.		Cortical atrophy	—	—	6.8	
19	K. E.	43	Cortical atrophy	—	41	6.4	
20	W. M.	4	Hydrocephalus	—	—	4.8	
21	Ch.	2	Agenesis of corpus callosum	—	—	4.6	
22	S. N.	33	Strychnine poisoning	177	78	8.1	
23	W.	34	Syringomyelia	105	36	10.5	
Mean and standard deviation					47.7 ± 22.3	6.6 ± 1.4	
C. Neurologic Disorders Suspected to Be Caused by a Space Occupying Lesion But Ruled Out by Special Procedures							Diagnosis proved by:
24	D. L.	63	Epilepsia tarda	168	55	7.0	Operation
25	A. Ch.	49	Epilepsia tarda	90	38	5.9	Pneumoencephalogram
26	Ch. L.	23	Arnold-Chiari syndrome	120	85	6.7	Operation
27	G. Ch.	49	Arnold-Chiari syndrome	98	103	6.4	Operation
28	A. E.	29	Pseudotumor cerebri	—	35	7.0	Ventriculogram
29	D. M.	49	Astereognosis rt. side postmastectomy for breast cancer	109	26	5.8	Pneumoencephalogram, myelogram

TABLE I.—CONT'D

NUMBER	PATIENT	AGE	DIAGNOSIS	SERUM MUCOPROTEIN (MG. %)	CEREBROSPINAL FLUID		
					PROTEIN (MG. %)	MUCOPROTEIN (MG. %)	REMARKS
30	T. F.	43	Severe headaches	84	83	6.8	Pneumoencephalogram
31	L. Ch.	38	Severe headaches	—	28	4.8	Pneumoencephalogram
32	K.	24	Headaches post-concussion	75	71	5.3	Pneumoencephalogram
33	M. A.		Herniated discus, cervical	—	54	6.5	Operation
34	K. V.	49	Herniated discus, lumbar	126	184	5.5	Myelogram
35	M. R.	50	Organic mental syndrome	—	95	6.1	Pneumoencephalogram
Mean and standard deviation					71.4 ± 44.2	6.2 ± 0.7	
D. Bacterial Infections of the Central Nervous System							
36	L. M.	26	Meningitis Ps. aeruginosa	177	230	14.4	
37	Z. Z.	4	Brain abscess	277	116	13.5	Operation
38	D. S.	68	Subacute bact. endocarditis				
			Meningoencephalitis	190	171	15.4	Postmortem
39	Ch.	75	Septicemia: meningitis	314	94	13.7	Postmortem
Mean				239.5	152.5	14.3	
E. Tumors							
40	L. R.	38	Ependymoma	141	156	13.6	Operation
41	K. E.	45	Hemangioendothelioma	98	556	17.1	Postmortem
42	Ap.	61	Astrocytoma	172	60	11.2	Operation
43	I.	49	Astrocytoma	—	442	11.2	Operation
44	B. A.	19	Craniopharyngioma	—	240	12.6	Operation
45	L.		Ca thyroid, brain metastases	167	396	10.1	Ventriculogram
46	Y.	59	Glioma	198	120	14.2	Postmortem
47	K.	42	Tumor of petrous bone	212	106	15.2	Operation
Mean and standard deviation				164.7	259.5	13.2 ± 2.2	
F. Clinically Diagnosed Tumors							
48	A. F.	40	Epilepsia tarda	102	27	12.4	
49	K. M.	61	Spinal tumor	126	84	11.6	

To determine the mucoprotein fraction, which is normally present only in low concentrations in the cerebrospinal fluid, a modification of Weimer and Moshin's method[8] was used. This modified procedure was carried out as follows. To 5 ml. of CSF or to 0.5 ml. of serum diluted with 4.5 ml. of saline, add dropwise during a period of 40 seconds 2.5 ml. of 1.8 M perchloric acid. Mix and let stand at room temperature for 10 minutes. Filter through a dry Whatman No. 50 filter paper. The precipitated CSF must be filtered twice in order to obtain a clear solution. To an aliquot of 5 ml. of the filtrate in a centrifuge tube add 1 ml. of 5 per cent solution of phosphotungstic acid in 2 N HCl, let stand for 10 minutes at room temperature, then centrifuge at 2,000 r.p.m for 10 minutes. Decant, dry the tube with filter paper and add to it 5 ml. of 0.2 N sodium hydroxide. Prepare a blank (5 ml. 0.2 N NaOH) and a standard (5 ml. of 0.2 N NaOH containing 1 mg. casein). Add 1 ml. of Mehl's[9]

biuret reagent diluted 1:5 with 0.2 N NaOH. Close with a rubber stopcork and agitate thoroughly. Keep in the dark for one hour and read at 300 or 320 mμ with a Beckman DU spectrophotometer. The calculation of the quantity of the seromucoid is carried out as follows:

$$\frac{\dfrac{\text{Absorbance of unknown}}{\text{Absorbance of standard (1 mg. casein)}} \times 100}{\dfrac{\text{Vol. of filtrate used for determination (5 ml.)}}{\text{Total vol. of filtrate}} \times \dfrac{\text{Vol. of serum}}{\text{or C.S.F.}}} = \text{mg. \%}$$

RESULTS

The results of the determination of the mucoprotein fraction in the serum and cerebrospinal fluid and of the total proteins of the cerebrospinal fluid are presented in Table I. Since some neurologic cases included in this study had a systemic infection known to raise the serum mucoproteins, the mean and standard deviation of the mucoprotein fraction has been calculated for the cerebrospinal fluid only. The mean and standard deviation of the serum mucoproteins in the 12 control cases was 74.2 ± 10.4 mg. per cent and that of the cerebrospinal fluid mucoproteins in the 8 controls 5.1 ± 1.8 mg. per cent. The results indicate clearly that the cerebrospinal fluid mucoprotein content is increased in infections (mean 14.3 mg. per cent) and in tumors (mean 13.2 ± 2.2 mg. per cent) of the central nervous system. In other disorders of the central nervous system the mucoproteins of the cerebrospinal fluid appear to be only slightly increased (mean 6.6 ± 1.4 mg. per cent). Only in one case of syringomyelia was the cerebrospinal fluid mucoprotein content high (10.5 mg. per cent). No direct correlation between the mucoprotein level of the serum and that of the cerebrospinal fluid could be found. Cases in which the serum mucoproteins were increased because of pulmonary or urinary infection had a normal cerebrospinal fluid mucoprotein level (Cases 10, 12, and 14), while cases with increased cerebrospinal fluid mucoproteins had a normal or only slightly increased serum mucoprotein level (Cases 3, 4, and 41). Only in tumors and infections of the central nervous system was the mucoprotein level elevated both in the serum and in the cerebrospinal fluid. No clear correlation could be found between the cerebrospinal fluid total protein content and the mucoprotein level. In tumors and infections of the central nervous system both the total proteins and the mucoprotein content were increased. In other neurologic disorders, although the total proteins were high in some cases (Cases 11, 27, and 34), the mucoprotein level was within the range or not much above that of the controls.

DISCUSSION

The mucoprotein content of the serum is known to be high in infections, tumors after experimental irradiation, and in myocardial infarction.[10] In neurologic disorders the total blood glycoproteins were found slightly elevated in tumors of the central nervous system.[11] Assuming that the pathologic changes due to diseases of the central nervous system were better reflected in

the cerebrospinal fluid than in the blood, Apostol and associates[12] examined the total glycoprotein content of the cerebrospinal fluid, and indeed found it increased in tumors of the central nervous system. Individual variations were however so great that the standard deviation was superior to the mean (controls 14.4 ± 4, brain tumors 42.8 ± 51.5). The practical value of the determination of the total glycoproteins in the cerebrospinal fluid with regard to the diagnosis of tumors of the central nervous system seems, therefore, questionable. Since the glycoproteins do not form a homogenous group, and as the levels of the total glycoproteins and the mucoproteins might vary independently,[3] we preferred to examine the mucoprotein fraction only. It was found that the mucoprotein level in the cerebrospinal fluid was high in cases of tumors and infections of the central nervous system. The individual variations were rather small. The highest value of the mucoprotein content found in cases of general neurologic disorders did not reach the lowest determined in tumors of the central nervous system, except in one case of syringomyelia.

The origin of the increased mucoprotein level in the cerebrospinal fluid is not clear. Apostol and associates[12] suggested that an increase of the glycoproteins in the cerebrospinal fluid might be due to an overflow from elevated blood glycoproteins. This cannot hold true for the mucoprotein fraction, since cases presenting a high serum mucoprotein level were found to have a normal cerebrospinal mucoprotein content. The origin of the increased mucoprotein level in the cerebrospinal fluid, in tumors, or in bacterial infections of the central nervous system may be related to the metabolic activity taking place at the site of the tumor or infection. That mucoproteins may be produced by such a process in other tissues has been shown by Pearse,[13] who found an intracellular mucoprotein in the plasma cells surrounding growing tumors. Zlotnick and co-workers[14] found an elevation of the mucoprotein level in the serum of rabbits immunized with *Salmonella typhosa*, as well as mucoprotein-storing cells in their spleens. Since it has been established that hyaluronic acid is found in the axoplasma of peripheral nerves and that sialic acid is a major constituent of brain gangliosides,[15] it may be assumed that tumors or bacteria acting on the nervous tissue give rise to mucoproteins which appear in the cerebrospinal fluid.

According to the results arrived at in this study a high level of mucoproteins in the cerebrospinal fluid should raise the suspicion of a tumor of the central nervous system in cases where an acute infection or an abscess of the central nervous system can be ruled out. If further studies, carried out on a greater number of patients, confirm these results, the determination of the cerebrospinal fluid mucoprotein content may become an important auxiliary test for the diagnosis of tumors of the central nervous system.

SUMMARY

The mucoprotein content of blood serum and cerebrospinal fluid was determined in 49 neurologic patients and 8 control subjects.

The cerebrospinal fluid mucoprotein level was found elevated in tumors and infections of the central nervous system.

No clear correlation was established between the blood and cerebrospinal fluid mucoprotein levels, nor between the cerebrospinal fluid protein and mucoprotein content.

The origin of the increase in mucoprotein in the cerebrospinal fluid is discussed.

We wish to thank Prof. L. Halpern and Prof. J. J. Groen for their advice and constructive criticism, and Dr. E. Peyser, of the Rambam Hospital, Haifa, for the cerebrospinal fluid of Cases 13 and 46.

REFERENCES

1. Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, J. M.: Studies on the Mucoproteins of Human Plasma. I. Determination and Isolation, J. Clin. Invest. **27:** 609, 1948.
2. Greenspan, E. M.: Clinical Significance of Serum Mucoproteins, Advances Int. Med. **7:** 101, 1955.
3. Winzler, R. J.: Methods of Biochemical Analysis, edited by David Glick, New York, 1955, Interscience Publishers, Inc., vol. 2, p. 279.
4. Stary, Z.: Mucoproteins in Clinical Chemistry, Clin. Chem. **3:** 557, 1957.
5. Roboz, E., Apostol, R. R., Luft, A. M., and Hess, W. C.: Determination of the Carbohydrates in the Glycoproteins and Mucoproteins of the Cerebrospinal Fluid, J. Lab. & Clin. Med. **49:** 796, 1957.
6. Stary, Z., Bodur, H., and Lisie, S. G.: Über den Kohlenhydratgehalt der Liquorproteine, Klin. Wchnschr. **31:** 339, 1953.
7. Lowry, O. H., Rosebrough, N., and Farr, A.: Protein Measurement With the Folin Phenol Reagent, J. Biol. Chem. **193:** 265, 1951.
8. Weimer, H. E., and Moshin, J. R.: Serum Glycoprotein Concentrations in Experimental Tuberculosis of the Guinea Pig, Am. Rev. Tuberc. **68:** 594, 1953.
9. Mehl, J. W.: The Biuret Reaction of Proteins in the Presence of Ethylene Glycol, J. Biol. Chem. **157:** 173, 1945.
10. Weimer, H. E., Moshin, J. R., Quinn, F. A., and Nelson, E. L.: Serial Determination of Serum Glycoproteins Following Antigen Injection Into Previously Immunized Animals, J. Immunol. **78:** 1, 1957.
11. Roboz, E., Hess, W. C., and Forster, F. M.: Determination of Serum Proteins and Polysaccharides. Comparison of Neoplastic Diseases Particularly of the Central Nervous System, A. M. A. Arch. Neurol. & Psychiat. **73:** 536, 1955.
12. Apostol, R. R., Roboz, E., Hess, W. C., and Forster, F. M.: Changes in the Glycoproteins of the Cerebrospinal Fluid in Neurological Diseases, Neurology **6:** 859, 1956.
13. Pearse, A. G. E.: The nature of Russell Bodies and Kurloffs Bodies: Observations on the Cytochemistry of Plasma Cells and Reticulum Cells, J. Clin. Path. **2:** 81, 1949.
14. Zlotnick, A., Gerichter, C. B., and Nir, J.: Experimental Production of "Grape Cells" and Their Relation to the Serum Globulins and Seromucoids, Blood **14:** 564, 1959.
15. Blix, F. G.: General Discussion in CIBA Foundation Symposium on the Chemistry and Biology of Mucopolysaccharides, London, 1958, J. & A. Churchill, Ltd., p. 308.

DIURNAL VARIABILITY OF SERUM CHOLESTEROL AT NORMAL AND REDUCED LEVELS

WILLIAM SHAPIRO, M.D.,* E. HARVEY ESTES, JR., M.D., AND
HELEN L. HILDERMAN, A.B.
DURHAM, N. C.

IN RECENT years, serum cholesterol has been effectively lowered in human subjects by a variety of diets and chemicals. It has been shown that reduced levels may be maintained for weeks or months, but the extent and pattern of hour-to-hour fluctuations once reduction has been obtained remain speculative.

Some studies are available dealing with normocholesteremic subjects and appear to agree on the following points:

1. Variations in serum cholesterol in the course of a single day are limited to within approximately 10 per cent of the individual's mean value for the day.[1-6]

2. In most experiments, the small fluctuations that have been observed are not related to meals, activity, or time of day. Instead, they appear to be inconsistent and casual.

3. A blood sample drawn at any time during the day will yield values closely approximating the individual's mean for the day.

The purpose of this study was to document the extent and pattern of the hourly fluctuations exhibited by the serum total cholesterol in a group of apparently healthy normocholesteremic subjects whose levels had been significantly reduced by dietary means, in contrast with a control group of similar subjects partaking of a normal diet.

METHODS

Ten healthy, active men house staff officers and medical students, 23 to 30 years old, were studied. For several weeks prior to the observation period, 4 subjects had been on a modified rice-fruit diet, supplemented only by salt ad libitum and a maximum of 15 Gm. of fat as butter per day. The remaining 6 subjects had been eating their customary normal diet (what has become popularly termed "the average American diet"), i.e., about 3,000 calories, 100 Gm. protein, and 120 to 150 Gm. fat, largely of animal origin, per day.

On the day an individual was studied, the first sample was drawn in the morning after a 12- to 14-hour overnight fast. Succeeding samples were drawn hourly through an indwelling Cournand needle until the subject retired for the night. Meals and activities during this period were carried on as usual; i.e., they continued with their hospital duties, and either rice or normal diets were eaten as in the preobservation period.

From the Department of Medicine, Veterans Administration Hospital, and the Duke University School of Medicine, Durham, N. C.

Part of this study supported by a grant from the Greensboro Heart Association through the North Carolina Heart Association.

Received for publication Sept. 30, 1958.

*United States Public Health Service. Research Trainee of the National Heart Institute, Bethesda, Md.

Total serum cholesterol values were obtained in duplicate by the direct method of Pearson, Stern, and McGavack.[7]

RESULTS

The results are summarized in Table I.

TABLE I. HOURLY CHOLESTEROL VALUES FOR EACH OF TEN SUBJECTS

SUBJECTS ON MODIFIED RICE-FRUIT DIET				SUBJECTS ON NORMAL DIET					
A	B	C	D	1	2	3	4	5	6
142	142	153	149	231	241	226	254	186	196
143	141	149	152	231	235	210	246	196	199
136	138	138	146	234	235	197	241	202	197
136	145	147	153	239	231	210	247	199	198
138	142	159	148	236	227	202	244	194	196
135	139	153	140	231	229	203	252	176	213
139	142	155	142	240	227	201	246	188	207
147*	146	151	145	238	216	200	243	197	192
128*	139	155	134	242	221	221	237	191	195
135*	139	165	148	236	225	208	248	195	215
	138	159*	139	230	223	215	252	196	211
	132	147*	143	226	224	215	245	196	216
	127	145*	129	221	220	214	252	194	225
	126		140	229	213	219	239	188	214
	126		133	220	208	222	251		214
	120		146	224	209	221			214
					222	203			
Mean 137.9	136.4	152.0	142.9	231.8	223.9	211.3	246.5	192.7	206.4
S.D. ±5.3	±7.7	±7.0	±6.8	±6.7	±9.1	±8.8	±5.1	±6.5	±10.1
Mean S.D. = ±6.7				Mean S.D. = ±7.7					
Mean cholesterol = 142.3				Mean cholesterol = 218.8					

*Samples at 2-hour intervals.

The mean total cholesterol of the 4 subjects on the modified rice-fruit diet had been 202 mg. per cent prior to the period of dietary restriction. On the day studied, their mean was 142.3 mg. per cent. The drop in serum cholesterol in the 4 subjects on the rice-fruit diet ranged from 46 to 70 mg. per cent. This change was significant at the 1 per cent probability level. The drop in serum cholesterol occurred in the first 10 days of the diet, and was maintained at approximately the same level for the 2 weeks prior to the study.

The mean value of all the cholesterol determinations made in the normal group on the day of the study was 218.8 mg. per cent. Despite large differences in the group and individual means, the standard deviation of the hourly fluctuations for the groups as well as for the individuals is of similar magnitude. In the normal diet group, the minimum and maximum S.D. were ± 5.1 mg. per cent and ± 10.1 mg. per cent, respectively, while for the rice diet group these figures were ± 5.3 mg. per cent and ± 7.7 mg. per cent, respectively. The mean S.D. was 6.7 mg. per cent for the rice group (or 4.8 per cent of mean total cholesterol) and 7.7 mg. per cent for the normal group (or 3.5 per cent of mean total cholesterol). Thus, the spread of each individual's hourly values about his mean for the day was relatively narrow (95 per cent of values were within 10 per cent of the mean). In each instance, however, the S.D. was greater than the error of the method, ± 3.48 mg. per cent. Therefore, many of the hourly fluctuations observed cannot be discounted as simply being due to technical error alone.

Despite these individual trends, there appeared to be no consistent pattern of change in relation to meals, activity, or time of day. The most striking observation was that the reduced values remained low—never in the course of the observation period did they approach the previous normal levels (195 to 212) that each of these individuals had possessed prior to his change in diet. An analysis of variance performed on the data from each group demonstrated that the variations in values are largely due to the differences between individuals in each group, and that these differences were significantly greater than the fluctuations with time exhibited by the members of each group. This was somewhat more pronounced in the data from the normal group, but the F test value was < 1 per cent for each group.

CONCLUSIONS

From the data presented, we may conclude that during any one waking period, the cholesterol regulatory mechanism maintains the blood level of normocholesteremic subjects within a narrow range independent of activity or dietary intake encountered during this study, which approximates "normal" activity and diet. Further, the attainment (by means of a rice-fruit diet, at least) of a reduced level is characterized by the maintenance of that level, within a narrow range, during the course of a given day. It is presumed that hypocholesterolemia produced by other means would also show this stable quality. Hourly fluctuations during periods of active change in the mean total cholesterol level may well show much wider fluctuations than observed under the relatively stable conditions of this study, but this is speculative.

The small variations from the mean that were observed at the reduced values were of the same order of magnitude as were the variations at normal levels. The majority of the hourly values were within 5 per cent of the mean value for the day, and 95 per cent of them within 10 per cent.

Variability of this slight degree implies (with the possible exception of periods of rapid change in levels) that the serum total cholesterol on any one day need not be drawn at a particular time in order to obtain a close estimate of the mean value for that day, whether the value be normal or at reduced levels.

REFERENCES

1. McClure, C. W., and Huntsinger, M. E.: Studies in Fat Metabolism. I. The Influence on Blood Lipids of Single Foodstuffs, J. Biol. Chem. **76**: 1, 1928.
2. Bruger, M., and Somach, I.: The Diurnal Variations of the Cholesterol Content of the Blood, J. Biol. Chem. **97**: 23, 1932.
3. Boyd, E. M.: Diurnal Variations in Plasma Lipids, J. Biol. Chem. **110**: 61, 1935.
4. Sperry, W. M.: The Concentration of Total Cholesterol in the Blood Serum, J. Biol. Chem. **117**: 391, 1937.
5. Turner, K. B., and Steiner, A.: A Long Term Study of the Variation of Serum Cholesterol in Man, J. Clin. Invest. **18**: 45, 1939.
6. Chandler, H. L., Lawry, E. Y., Potee, K. G., and Mann, G. V.: Spontaneous and Induced Variations in Serum Lipoproteins, Circulation **8**: 723, 1953.
7. Pearson, S., Stern, S., and McGavack, T. H.: A Rapid, Accurate Method for the Determination of Total Cholesterol in Serum, Anal. Chem. **25**: 813, 1953.

ALTERATIONS IN RESPIRATORY FUNCTION DURING NATURAL SLEEP

RICHARD I. BIRCHFIELD, M.D.,* HEBERT O. SIEKER, M.D., AND
ALBERT HEYMAN, M.D.
DURHAM, N. C.

ALTERATIONS in pulmonary ventilation and patterns of respiration during sleep have been recognized for many years. Endres[1] found an elevation of carbon dioxide tension and DeBruin[2] commented on the decrease in respiratory rate noted with sleep. Alterations in patterns of breathing, such as Cheyne-Stokes respiration, are frequently observed in normal sleeping subjects. More recently Robin and colleagues[3] reported a reduction in pulmonary ventilation and altered sensitivity of the respiratory center. These workers used alveolar gas tensions as a measure of arterial blood gases.

Previous work in our laboratory demonstrated that a retention of carbon dioxide and a slight decrease in arterial oxygen saturation appeared with drowsiness and remained unchanged during deep sleep.[4] Ventilatory studies, however, were not done and the explanation for the alterations in blood gases was speculative. In the present investigation the changes in ventilation during sleep were correlated with the arterial blood pH, oxygen saturation, and carbon dioxide tension. In addition the ventilatory response to carbon dioxide inhalation during sleep was observed.

MATERIALS AND METHODS

The respiratory changes in sleep were studied in 11 male volunteers ranging in age from 21 to 30 years. Nine were normal healthy students; the remaining two were convalescing hospital patients without respiratory disease or organic brain damage. Sleepiness was produced in the subject by early morning arousal, the studies usually beginning about 5 hours later at approximately 9 A.M.

Initial samples of air and blood were collected from each subject while he was awake and after being supine for at least 15 minutes. The volume of expired air was measured during periods of 3 to 4 minutes and a blood sample was taken during this time over a 30- to 60-second period. Air samples were obtained in Douglas collecting bags connected to a face mask firmly attached about the subject's nose and mouth. Care was taken to avoid leakage about the mask. The dead space of the system was approximately 60 ml. Arterial blood samples were taken in oiled heparinized syringes from an indwelling Cournand needle inserted into the brachial artery and connected to a manifold by polyethylene tubing.

From the Department of Medicine (Neurology and Pulmonary Divisions), Duke University Medical Center, and the Veterans Administration Hospital, Durham, N. C.

Supported by Research Grant B669, National Institutes of Health, United States Public Health Service, and by a grant from the American Heart Association.

Received for publication Nov. 18, 1958.

*Present address: Division of Neurology, Department of Medicine, University of Washington School of Medicine, Seattle, Wash.

Following collection of the initial samples the room was darkened and quieted and the subject allowed to fall asleep. Most of the individuals slept for periods of at least 10 to 15 minutes. The state of wakefulness or sleep was determined by a continuous electroencephalographic record. Respiratory rhythm was monitored by a pneumograph synchronously recorded with the electroencephalogram. Only subjects with regular breathing patterns during sleep were studied to avoid the blood gas changes occurring with periodic respirations. During sleep, repeat samples of blood and expired air were obtained, following which the subjects were given a mixture of 5 per cent carbon dioxide, 21 per cent oxygen and 74 per cent nitrogen instead of room air. Only 4 of the subjects continued to sleep while breathing this mixture. The remaining subjects aroused spontaneously or were awakened by the carbon dioxide mixture, usually with the sensation of dyspnea. Samples of blood and expired air were collected from the sleeping individuals at the end of 5 minutes of CO_2 inhalation. Additional samples were taken approximately 10 minutes after arousal before any change in position occurred. These together with the initial samples, were used as control values for the awake state. The studies on the ventilatory response to CO_2 inhalation were repeated after arousal in the 4 subjects who were able to sleep during this procedure.

The volume of expired air was measured and converted to standard temperature and pressure. Blood oxygen content and saturation were determined by the photometric method of Hickam and Frayser.[5] Blood carbon dioxide was determined by the method of Van Slyke and Neill.[6] The pH of whole blood was measured with a Cambridge model R pH meter with enclosed glass electrode; measurements were corrected to 37° C. using Rosenthal's factor.[7] Plasma carbon dioxide content was calculated from the blood carbon dioxide content, pH, and hemoglobin using the line chart of Van Slyke and Sendroy[8]; carbon dioxide tension was calculated from this value using the Henderson-Hasselbalch equation with a pK of 6.11. All determinations were done in duplicate.

RESULTS

The changes in arterial pH, blood gases, and ventilation during sleep are shown in Table I. While awake, the mean arterial carbon dioxide tension in the 11 subjects was 45.6 mm. Hg. This value was obtained after the face mask

TABLE I. CHANGES IN ARTERIAL BLOOD AND PULMONARY VENTILATION DURING SLEEP

	BLOOD O_2 (% SAT.)		BLOOD pCO_2 (MM. Hg)		BLOOD pH		MINUTE VOLUME (L./MIN.)		RESPIRATIONS/MIN.		TIDAL VOLUME (ML.)	
SUBJECT	C	S	C	S	C	S	C	S	C	S	C	S
A. P.	95	93	51	53	7.325	7.305	4.6	3.9	14	15	328	260
G. S.	94	93	39	45	7.407	7.371	8.0	8.0	15	23	535	347
C. P.	93	93	48	50	7.358	7.334	6.9	6.8	20	21	345	324
W. L.	96	94	49	51	7.363	7.349	5.8	6.1	10	15	580	407
J. C.	96	96	46	51	7.374	7.341	8.2	8.0	15	17	547	470
L. W.	96	94	40	42	7.400	7.384	8.1	7.4	10	12	810	616
G. W.	97	94	38	46	7.419	7.358	8.0	6.3	14	16	570	394
J. B.	93	91	50	54	7.352	7.328	7.7	6.1	16	15	481	407
P. B.	95	96	47	52	7.346	7.323	7.8	7.7	12	20	650	385
J. S.	95	92	50	56	7.337	7.290	6.3	6.1	12	15	525	407
A. L.	96	95	44	47	7.362	7.340	8.3	6.5	19	16	437	406
Mean	95.1	93.7	45.6	49.7	7.368	7.338	7.2	6.6	14	17	528	402
p	< 0.01		< 0.001		< 0.001		< 0.05		< 0.05		< 0.001	

C = Control, awake; S = subject asleep. p = Statistically significant differences.

and valve system were applied to the awake subjects. It was slightly higher than the mean resting value of 44.4 mm. Hg obtained in the same subjects awake and unmasked. During sleep, there was a significant increase to a mean value

of 49.7 mm. Hg ($p < 0.001$) (Fig. 1). An elevation in carbon dioxide tension was observed in each subject usually coincident with drowsiness at the onset of sleep. In the awake state the mean arterial oxygen saturation was 95.1 per cent. During sleep, there was a slight decrease in all but 3 subjects, the mean value showing a significant reduction to 93.7 per cent ($0.01 > p > 0.001$). The arterial pH showed a significant change with the mean values falling from 7.368 while awake to 7.338 during sleep ($p < 0.001$).

The minute volume of expired air fell from a mean value of 7.2 L. while awake to 6.6 L. during sleep ($p < 0.05$). This reduction in ventilation occurred despite a mean increase in the respiratory rate from 14 respirations per minute while awake to 17 per minute during sleep. The percentile reduction in tidal volume (23.8 per cent), therefore, was considerably greater than the change in minute volume during sleep (8.3 per cent). With the dead space remaining constant, the alveolar ventilation was reduced even more than indicated by the decrease in minute ventilation.

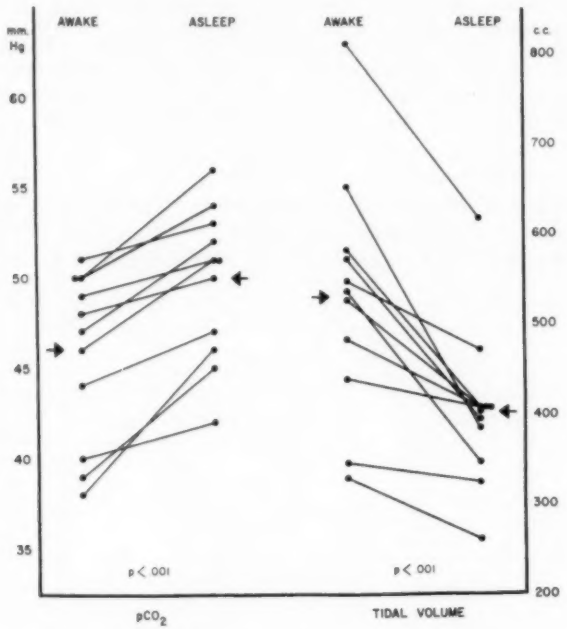


Fig. 1.—Changes in arterial carbon dioxide tension and tidal volume during sleep. The arrows indicate the mean values in each state of awareness.

The ventilatory response to 5 per cent carbon dioxide in the 4 subjects who slept throughout the inhalation of this gas mixture is shown in Table II. While these subjects were awake, carbon dioxide produced a slight increase in

TABLE II. EFFECTS OF CO_2 INHALATION ON ARTERIAL BLOOD AND PULMONARY VENTILATION WHILE AWAKE AND ASLEEP

SUBJECT	INSPIRED GAS	BLOOD O_2 (% SAT.)		BLOOD pCO_2 (MM. Hg)		BLOOD pH		RESPIRATIONS/MIN.		TOTAL VENT. (L./MIN.)		TIDAL VOLUME (ML.)	
		C	S	C	S	C	S	C	S	C	S	C	S
A. P.	Room air	95.1	92.7	51	53	7.325	7.305	14	15	4.6	3.9	328	260
	5% CO_2	97.5	96.3	56	60	7.310	7.270	15	15	10.5	7.5	700	500
C. P.	Room air	93.1	92.6	48	50	7.358	7.334	20	21	6.9	6.8	345	324
	5% CO_2	95.1	91.0	53	5[illegible]	7.314	7.312	22	24	12.7	11.5	578	480
J. C.	Room air	95.5	95.6	46	51	7.374	7.341	15	17	8.2	8.0	547	470
	5% CO_2	97.5	96.8	50	—	7.351	——	19	19	19.2	14.4	1010	760
J. S.	Room air	94.7	92.3	50	56	7.337	7.290	12	15	6.3	6.1	525	407
	5% CO_2	97.0	95.0	54	59	7.301	7.267	15	17	13.3	10.6	886	624
Mean	Room air	94.6	93.3	49	53	7.349	7.318	15	17	6.5	6.2	436	365
	5% CO_2	96.8	94.8	53	58	7.319	7.283	18	19	13.9	11.0*	794	591

C = Control, awake; S = subject asleep.

*Statistically significant difference ($p < 0.05$) from mean value in same subjects breathing CO_2 while awake.

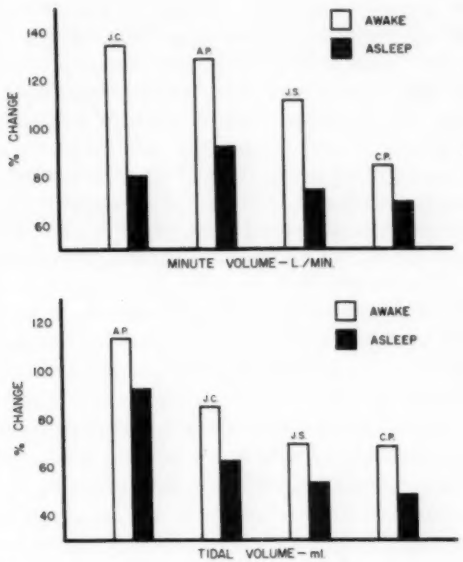


Fig. 2.—The ventilatory response of carbon dioxide inhalation in 4 subjects while awake and during sleep.

oxygen saturation (94.6 to 96.8 per cent), a rise in carbon dioxide tension (49 to 53 mm. Hg), and a fall in arterial pH. Similar changes in blood gases and pH were noted following the inhalation of this gas mixture during sleep. The ventilatory response to carbon dioxide inhalation in sleep, however, was less than that observed while awake (Fig. 2). In the awake state, the minute volume showed a mean increase from 6.5 to 13.9 L. During sleep, carbon dioxide

inhalation produced a mean increase in minute volume to only 11.0 L., a value significantly less than that observed in the same subjects while awake ($p < 0.05$). In both the asleep and awake states, there was an increase of 2 to 3 respirations per minute during carbon dioxide inhalation. The mean tidal volume while awake increased from 436 to 794 ml. during carbon dioxide inhalation. During sleep there was significantly less change with a mean increase to only 591 ml. ($p < 0.02$).

DISCUSSION

These data demonstrate that the elevation in arterial carbon dioxide in sleep is associated with a diminution in total ventilation and tidal volume. This reduction in ventilation occurs despite a slight rise in respiratory rate. Carbon dioxide inhalation during sleep also fails to produce as great an increase in ventilation as in the awake state. These findings have led to the suggestion that there is a depression of respiratory center sensitivity in sleep.[3] The fact that the alterations in blood gases appear early in sleep and that they are not altered by the depth of sleep would indicate that the respiratory center depression must be due to influences other than gradual retention of carbon dioxide or progressive hypoxia. Rapid alterations in neuronal activity are known to occur in the ascending reticular pathways in the brain stem during drowsiness and sleep,[9] and similar neurophysiologic mechanisms may affect respiratory function during these states of altered consciousness. Indeed, there is a close anatomic association of the respiratory center and the reticular system in the medulla, and inhibitory influences on respiration may arise from this general area.[10] A close interrelationship between sleep and inhibition of respiration has also been noted at higher cerebral levels. Kaada and Jasper[11] have shown that in man stimulation of the anterior end of the hippocampal gyrus, the medial surface of the temporal pole, and the anterior limbic gyrus have produced inhibition of respiratory movements with impairment of consciousness, a feeling of tiredness and sleepiness, and a tendency to close the eyes. These areas of the brain are not believed to be specific for respiratory function nor to be directly involved in sleep mechanisms, but they are probably part of the complex pattern related to these functions. Our previous studies showed that the changes in blood gases appear at the earliest electroencephalographic evidence of drowsiness, indicating that the stimuli of wakefulness are needed to maintain respiratory function at optimal levels.[4]

The decrease in ventilatory response to carbon dioxide inhalation during sleep is of considerable interest. A reduction in sensitivity to the carbon dioxide stimulus has been observed in chronic hypercapnia[12] and it is possible that the acute hypercapnia of sleep accounts for the observed decrease in respiratory center sensitivity. Conversely, the hypocapnia observed in individuals residing at high altitudes and following hyperventilation in a tank respirator is associated with increased CO_2 sensitivity.[13] It seems likely that the sensitivity of the respiratory center to carbon dioxide stimulus is in part a function of the prevailing blood carbon dioxide tension.

It should be emphasized that although 4 of our subjects had a decrease in respiratory center sensitivity to carbon dioxide inhalation during sleep, they showed nevertheless a 77 per cent increase in ventilation while breathing the carbon dioxide mixture. In 3 others the stimulus was sufficient to arouse the subject from sleep. It is apparent that although all of the sleeping subjects had retention of carbon dioxide, they did not respond uniformly to this stimulus. This suggests that the degree of decreased sensitivity of the respiratory center during sleep is variable.

The results obtained led to a study of the changes in respiratory function in other states of somnolence and in patients with decreased awareness caused by organic brain disease.[14] We have found that patients with narcolepsy often have hypercapnia and hypoxemia while awake and many of them, particularly those with moderate obesity, show a decrease in ventilatory response to carbon dioxide inhalation. On the other hand, patients with bilateral cerebral infarction show an increased sensitivity of the respiratory center to carbon dioxide inhalation. These observations suggest that alterations in the respiratory center sensitivity are produced through neuronal pathways as well as by changes in arterial carbon dioxide tension.

It is of interest that the 4 subjects who were able to sleep throughout carbon dioxide inhalation had a high pCO_2 mean value of 49 mm. Hg while awake, whereas the 7 remaining fatigued subjects who were able to sleep only during the ventilation study had a mean pCO_2 of 44 mm. Hg. These findings tend to confirm the observation of Mangold and his associates, who found that fatigued subjects who were able to sleep throughout their experimental procedures, had significantly higher presleep values of carbon dioxide tension than those subjects who were unable to do so.[15] These workers, however, did not find any alterations in blood gases during sleep per se.

CONCLUSIONS

1. The alterations in pulmonary ventilation, arterial blood gases, and pH and the ventilatory response to carbon dioxide have been determined in normal subjects during sleep.

2. There is a reduction in minute ventilation, a significant rise in carbon dioxide tension and a slight fall in arterial oxygen saturation and pH. A decrease in sensitivity to carbon dioxide stimulus also appeared during sleep.

3. It is suggested that the decreased ventilatory response to carbon dioxide stimulus in sleep is related not only to the prevailing level of blood gases but also to alterations in neuronal function. The decrease in respiratory function in sleep, however, is probably due to the absence of external stimuli which in the awake state maintains a higher rate of ventilation.

We are indebted to Mrs. Mary Pike and Miss Corinna Thomas for their valuable technical assistance.

REFERENCES

1. Endres, G.: Atmungsregulation und Blutreaktion im Schlaf, Biochem. Ztschr. **142**: 53, 1923.

2. DeBruin, M.: Respiration and Basal Metabolism in Childhood During Sleep, Acta paediat. **279**: 18, 1936.
3. Robin, E. D., Whaley, R. D., Crump, C. H., and Travis, D. M.: Alveolar Gas Tensions, Pulmonary Ventilation and Blood pH During Physiologic Sleep in Normal Subjects, J. Clin. Invest. **37**: 981, 1958.
4. Birchfield, R. I., Sieker, H. O., and Heyman, A.: Alterations in Blood Gases During Natural Sleep and Narcolepsy, Neurology **8**: 107, 1958.
5. Hickam, J. B., and Frayser, R.: Spectrophotometric Determination of Blood Oxygen, J. Biol. Chem. **180**: 457, 1949.
6. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry, Methods, Baltimore, 1932, Williams & Wilkins Company, vol. 2.
7. Rosenthal, T. B.: Effect of Temperature on pH of Blood and Plasma In Vitro, J. Biol. Chem. **173**: 25, 1948.
8. Van Slyke, D. D., and Sendroy, J., Jr.: Studies of Gas and Electrolyte Equilibria in Blood; Line Charts for Graphic Calculations by Henderson-Hasselbalch Equation, and for Calculating Plasma Carbon Content From Whole Blood Content, J. Biol. Chem. **79**: 781, 1928.
9. Magoun, H. W.: The Waking Brain, Springfield, Ill., 1958, Charles C Thomas, Publisher.
10. Brodal, A.: The Reticular Formation of the Brain Stem, Anatomical Aspects and Functional Correlations, Springfield, Ill., 1956, Charles C Thomas, Publisher.
11. Kaada, B. R., and Jasper, H.: Respiratory Responses to Stimulation of Temporal Pole, Insula and Hippocampal and Limbic Gyri in Man, A. M. A. Arch. Neurol. & Psychiat. **68**: 609, 1952.
12. Alexander, J. K., West, J. R., Wood, J. A., and Richards, D. W.: Analysis of the Respiratory Response to Carbon Dioxide Inhalation in Varying Clinical States of Hypercapnia, Anoxia, and Acid-Base Derangement, J. Clin. Invest. **34**: 511, 1955.
13. Brown, E. B., Jr.: Physiological Effects of Hyperventilation, Physiol. Rev. **33**: 451, 1953.
14. Heyman, A., Birchfield, R. I., and Sieker, H. O.: Effects of Bilateral Cerebral Infarction on Respiratory Center Sensitivity, Neurology **8**: 694, 1958.
15. Mangold, R., Sokoloff, L., Conner, E., Kleinerman, J., Therman, P. G., and Kety, S.: The Effects of Sleep and Lack of Sleep on the Cerebral Circulation and the Metabolism of Normal Young Men, J. Clin. Invest. **34**: 1092, 1955.

EXCRETION OF ACID MUCOPOLYSACCHARIDES IN THE URINE OF PATIENTS WITH MALIGNANT NEOPLASTIC DISEASES

CLAYTON RICH, M.D., AND W. P. LAIRD MYERS, M.D.
NEW YORK, N. Y.

ACID mucopolysaccharides (APS), among the best-defined constituents of ground substance of connective tissue, have been demonstrated also in leukocytes and serum.[1-6] The rates of excretion of APS in urine have been studied to see if they reflect the metabolic state of those tissues of the body which contain APS. Thus, it has been shown that amounts of APS excreted in urine of children can be correlated roughly to their rates of growth,[7] and that adults with diseases such as lupus erythematosus and rheumatoid arthritis (which are characterized by abnormal connective tissue metabolism) excrete increased amounts of APS.[8, 9] Work here reported shows that subjects with disseminated malignant diseases, particularly those with leukemia, have increased urinary excretion rates of APS.

MATERIALS AND METHODS

Twenty-nine subjects with disseminated malignant disease were studied. All were patients on the chemotherapy ward of the James Ewing Hospital. Diagnosis was confirmed by microscopic examination of material removed by biopsy or during an autopsy. Usually the APS concentration of 2 or more 24-hour urine collections from each patient was measured in duplicate by a method previously described.[10] The APS concentration of many of the samples was determined before and after dialysis to demonstrate that the material measured would not pass by dialysis through a cellophane membrane.

In addition APS was precipitated from 1 to 4 L. of urine of 6 subjects by cetyltrimethylammonium bromide. The precipitate was washed with ethanol, dried, dissolved in phosphate buffer, and incubated with trypsin. The remaining protein was precipitated with phosphotungstic acid, and the supernate dialyzed and dried over P_2O_5. This procedure is a slight modification of that previously described.[11] Samples were analyzed for glucuronic acid, hexosamine, and sulfur by methods previously used,[11] and for neutral sugar by the anthrone reaction.[12] Neutral sugar content, expressed as galactose, was corrected for color yield from APS known to be present in the samples. Glucosamine and galactosamine of hydrolyzed samples were measured after paper chromatographic separation of the aminosugars in a pyridine-butanol-water solvent.[13] Part of each chromatogram was stained with ninhydrin or by modification of the Elson-Morgan reaction.[14] The remainder was cut into strips parallel to the solvent front, eluted, and the hexosamine from each strip determined quantitatively.

Chromatography of each sample of APS was carried out in a 35 per cent n-propanol citrate buffer (0.037 M, pH 4.1) at 2° C. These chromatograms also were divided into

From the Rockefeller Institute and Division of Clinical Chemotherapy, Sloan-Kettering Institute for Cancer Research; the Department of Medicine, Memorial Center; and the Department of Medicine, Cornell University Medical College, New York, N. Y.

This study was supported in part by Grant C-1889 from the National Cancer Institute, United States Public Health Service, the American Cancer Society, the Damon Runyon Memorial Fund for Medical Research, and the Lasker Foundation.

Received for publication Nov. 21, 1958.

TABLE I. ACID MUCOPOLYSACCHARIDE (APS) EXCRETION RATES OF SUBJECTS WITH DISSEMINATED MALIGNANT NEOPLASTIC DISEASES

DIAGNOSIS	SEX	AGE	MEAN APS EXCRETION RATES (MG. GLUCURONIC ACID/DAY) DECREASED	NORMAL	INCREASED	GREATLY INCREASED	TREATMENT
Arbitrary classification of APS excretion rates	Male Female	Adult Adult	<4.5 <2.7	4.6-7.5 2.8-5.4	7.6-14 5.5-12	>14 >12	
I. Carcinoma							
A. Adenocarcinoma							
breast	F	63		4.9			Phosphonitrile
breast	F	48		2.9			Fumagillin
breast (1)*	F	68		4.9			Phosphonitrile, prednisone
breast and prostate	M	89		6.6			- - - - - -
sigmoid	F	73		3.9			- - - - - -
breast	F	54			7.8		- - - - - -
prostate (2)	M	76			11.8		X-ray, ethinyl estradiol
B. Other carcinoma							
anaplastic, lung	F	55		5.3			DON,† adenine
epidermoid, alveolar ridge	M	65			9.7		6-mercaptopurine glucoside
epidermoid, lung	M	52			11.1		DON, 6-mercaptopurine
epidermoid, piriform fossa	M	66			10.3		Epoxypiperazine
oat cell, lung	M	62			10.8		Epoxypiperazine
undifferentiated (melanoma?)	M	21			9.0		DON
II. Leukemia							
acute myeloblastic	M	17	3.2				X-ray, triamcinolone
acute myelomonocytic	M	43			13.5		6-mercaptopurine
acute myeloblastic	M	22			11.9		5-fluorouracil, prednisone
subacute myeloid	M	57			12.6		Amethopterin
chronic lymphatic	M	54			13.2		Triethylene melamine
acute myelomonocytic	M	58				33.5	6-chloropurine, prednisone
acute myeloblastic (3)	M	46				15.6	6-chloropurine
chronic lymphatic	M	58				17.8	Prednisone
chronic granulocytic, acute phase (4)	F	67				20.8	6-mercaptopurine, prednisone
III. Miscellaneous							
multiple myeloma	F	48			7.6		Fumagillin
multiple myeloma	F	70				12.7	- - - - - -
multiple myeloma	M	66				14.7	Pyrazolopyrimidine
fibrosarcoma	M	31			11.3		Azathymine
reticulum cell sarcoma	F	56				34.1	Amethopterin
Hodgkin's disease	M	52				26.9	Phosphonitrile
carcinoid, stomach	M	52				17.8	X-ray, epoxypiperazine

*Number following diagnosis identifies subject in Table II.
†DON = 6-diazo-5-oxo-L-norleucine.

strips parallel to the solvent front. The samples were eluted with dilute NH_4OH (pH 9). The eluates were dialyzed and concentrated to permit measurement of glucuronic acid, total hexosamine, glucosamine, and galactosamine.

RESULTS

Patients were grouped according to the average daily acid mucopolysaccharide excretion rates into those with normal, elevated, and greatly elevated rates of excretion. The amount of APS excreted (expressed as milligrams of glucuronic acid per day) and important clinical data are listed in Table I. The classification of APS excretion rates is arbitrary. Normal rates are those which fall within the observed range of a previous study.[10] The upper limit of increased rates was selected to include almost all adult subjects so far reported with lupus or rheumatoid arthritis.[5, 9] The standard deviation from the mean APS content of urine from the same patient on different days was 3.5 mg. per day.

A clear-cut quantitative difference is shown between the excretion rates of subjects with cancer and those with leukemia, lymphoma, or multiple myeloma. Subjects with adenocarcinoma tended to excrete normal amounts while those with epidermoid carcinoma generally excreted moderately increased amounts of APS. In contrast, except for 1 of the 3 patients with acute myeloblastic leukemia who excreted APS at an abnormally slow rate, all patients with leukemia, lymphoma, or multiple myeloma had moderately or greatly increased amounts of APS in their urine. There seemed to be no consistent difference between the excretion rates of subjects with different forms of leukemia. Because of the small population studied, only the most general correlations were expected. No such correlation could be drawn between the severity or extent of disease or location of metastases and the rate of APS excretion.

The results of analysis of APS collected from urine of 2 subjects with carcinoma, 2 with leukemia, and, for contrast, 2 normal adults are indicated in Table II. The glucuronic acid concentration varied between 13 and 29 per cent of dry weight. Assuming that APS was the source of all the glucuronic acid present, the composition of the samples with respect to APS varied from 36 to 76 per cent. Galactosamine was present in somewhat less than molar proportions to glucuronic acid and constituted 10.8 to 16.7 per cent of the samples. These samples contained 3.0 to 6.7 per cent glucosamine and 9.0 to 15.5 per cent carbohydrate not accounted for by APS present. Sulfur was found in molar or slightly more than molar concentrations when compared with glucuronic acid. After hydrolysis and chromatography, each sample was demontrated to have 8 or more different amino acids.

At least 95 per cent of the glucuronic acid of each sample migrated at the solvent front during chromatography in a propanol-citrate buffer. This area was intensely metachromatic when stained with toluidine blue. Standards of chondroitin sulfate migrated at the same rate, whereas hyaluronic acid migrated more slowly and heparin remained at the origin. The hexosamine/

TABLE II. COMPOSITION OF SAMPLES OF ACID MUCOPOLYSACCHARIDE ISOLATED FROM THE URINE OF PATIENTS WITH DISSEMINATED MALIGNANT NEOPLASTIC DISEASES AND FROM NORMAL SUBJECTS

DIAGNOSIS	CASE NO.*	GLUCURONIC ACID (%)	GALACTOSAMINE (%)	GLUCOSAMINE (%)	SULFUR (%)	MOLAR RATIO (GLUCURONIC ACID = 1)	
						GALACTOSAMINE	SULFUR
Carcinoma, breast	1	13.3	11.8	3.3	3.4	0.97	1.3
Carcinoma, prostate	2	24.6	17.7	7.3	5.1	0.81	1.3
Leukemia, acute myeloblastic	3	21.4	11.8	5.3	-	0.60	-
Leukemia, granulocytic acute phase	4	27.5	18.1	7.1	4.1	0.72	0.9
Normal subject		18.8	15.5	4.9	3.1	0.91	1.0
Normal subject		28.5	18.2	6.7	4.2	0.72	0.9

*Case number identifies subject in Table I.

glucuronic acid ratios of the material eluted from the solvent front were between 0.84 and 1.11. Only galactosamine could be identified in this material by chromatography and subsequent staining for amino sugar.

DISCUSSION

Chemical analysis of APS collected from urine of patients with carcinoma or leukemia showed no significant difference from that of normal subjects. Interpretation of the data is complicated because each sample contained non-APS material. Our analysis shows that about 40 per cent of this non-APS material is carbohydrate and that its hydrolysate is rich in amino acids. Other samples prepared by similar methods also appear to contain non-APS material (as indicated by a high nitrogen concentration and presence of glucosamine and amino acids in the hydrolysate[9]). The isolation procedures used result in recovery of all urinary APS but also any acidic mucoprotein, whether part of an APS-protein complex, or other material resistant to the action of trypsin and soluble in phosphotungstic acid. When APS prepared by a similar procedure was further purified by incubation with papain and treatment with kaolin, it appeared to be composed mainly of chondroitin sulfate.[11] Furthermore, all of the glucuronic acid containing material of our samples had a chromatographic mobility different from those of heparin and hyaluronic acid but similar to that of chondroitin sulfate, no detectable glucosamine, and a galactosamine content which was usually approximately the same as that of glucuronic acid. Therefore, it is probable that the major APS present in the samples was chondroitin sulfate and that the glucosamine, amino acids, and excess sulfur were derived mostly from contaminating material. However, small amounts of other APS may have been present.

In contrast to normal and moderately increased urinary APS excretion rates of subjects with carcinoma, those with leukemia and allied diseases excreted strikingly increased amounts of APS. These amounts were often much greater than those previously observed in adult subjects.

Too little is known about the factors which govern the concentration of APS in serum and urine to permit definite interpretation of these results. It is possible that serum and urinary APS is derived from connective tissue or other APS containing tissues, and that increased excretion rates reflect altered or accelerated connective tissue metabolism. However, serum APS may be formed at some other site or sites, and changes in its concentration may occur in relation to other processes, for instance, those affecting serum mucoprotein concentration (known to be elevated in several states, including leukemia[15] in which urinary APS excretion is increased).

SUMMARY

1. The rate of acid mucopolysaccharide (APS) excretion in urine was measured for 29 subjects with disseminated malignant neoplastic diseases. Of 13 subjects with cancer, 6 excreted normal and the rest excreted increased

amounts of APS. Eight of 9 subjects with leukemia and all patients studied who had multiple myeloma or sarcoma excreted APS at increased or greatly increased rates.

2. Acid mucopolysaccharides isolated from the urine of several patients with cancer or leukemia were found to be composed mainly of material similar to chondroitin sulfate, but also to contain significant amounts of neutral sugar and amino acids. No difference could be measured by the techniques employed between samples of APS from urine of normal subjects, patients with cancer, and patients with leukemia.

REFERENCES

1. Kerby, G. P.: The Occurrence of Acid Mucopolysaccharides in Human Leukocytes and Urine, J. Clin. Invest. **34**: 1738, 1955.
2. Bassiouni, M.: The Estimation of Heparin and Similar Substances in Human Blood and Tissues Using a Combined Biological and Colorimetric Method With Paper Electrophoretic Studies, J. Clin. Path. **7**: 330, 1954.
3. Denko, C. W., and Priest, R. E.: Distribution of Radiosulfur, S^{35}, J. LAB. & CLIN. MED. **50**: 107, 1957.
4. Schiller, S., and Dewey, K. F.: Isolation of Chondroitin-Sulfuric Acid From Normal Human Plasma, Fed. Proc. **15**: 348, 1956.
5. Bollett, A. J., Seraydarian, M. A., and Simpson, W. F.: Acid Mucopolysaccharides in Normal Serum, J. Clin. Invest. **36**: 1328, 1957.
6. Deutsch, H. F.: Some Properties of Human Serum Hyaluronic Acid, J. Biol. Chem. **224**: 767, 1957.
7. Rich, C., Di Ferrante, N., and Archibald, R. M.: Acid Mucopolysaccharide Excretion in the Urine of Children, J. LAB. & CLIN. MED. **50**: 686, 1957.
8. Di Ferrante, N., Robbins, W. C., and Rich, C.: Urinary Excretion of Acid Mucopolysaccharides by Patients With Lupus Erythematosus, J. LAB. & CLIN. MED. **50**: 897, 1957.
9. Di Ferrante, N.: Urinary Excretion of Acid Mucopolysaccharides by Patients with Rheumatoid Arthritis, J. Clin. Invest. **36**: 1516, 1957.
10. Di Ferrante, N., and Rich, C.: The Determination of Acid Aminopolysaccharide in Urine, J. LAB. & CLIN. MED. **48**: 491, 1956.
11. Di Ferrante, N., and Rich, C.: The Mucopolysaccharide of Normal Human Urine, Clin. chim. acta **1**: 519, 1957.
12. Graff, M. M., Greenspan, E. M., Lehman, I. R., and Holechek, J. J.: Estimation of Nonglucosamine Polysaccharides of the Serum Proteins With the Anthrone Reagent, J. LAB. & CLIN. MED. **37**: 736, 1951.
13. Chargaff, E., Levine, C., and Green, C.: Techniques for the Demonstration by Chromatography of Nitrogenous Lipide Constituents, Sulfur-Containing Amino-Acids and Reducing Sugars, J. Biol. Chem. **175**: 67, 1948.
14. Block, R. J., Le Strange, R., and Zweig, G.: Paper Chromatography, A Laboratory Manual, New York, 1952, Academic Press, Inc., p. 89.
15. Moschides, E., Stefanini, M., Magalini, S. I., and Kistner, S. A.: Content and Composition of the Mucoprotein Fraction of Human Serum (Seromucoid) in Disease, With Special Reference to Hematologic Disorders, J. Clin. Invest. **37**: 127, 1958.

FREE AMINO ACIDS IN LEUKOCYTES FROM NORMAL AND LEUKEMIC SUBJECTS

G. Y. N. Iyer, M.Sc.
Trivandrum, India

LARGE quantities of amino acids were found in the blood of leukemic patients by Okada and Hayashi[1] who noted that the amino nitrogen content of blood seemed to depend more on the number of white cells than of the red cells. They found that the amino acid concentrations in the white cells were about 6 to 7 times those of plasma. Other investigators[2, 3] have also reported increased free amino nitrogen content of blood in leukemia.

Data on the individual amino acid content of leukocytes seem to be quite limited. Nour-Eldin and Wilkinson[4] have estimated the total free amino nitrogen and also glutamic acid, glycine plus serine, taurine, and β-aminoethylphosphate which constituted nearly two-thirds of the free amino nitrogen. They have also identified alanine, threonine, aspartic acid, and leucine in white cell extracts.

In the present investigation the author has studied qualitatively and quantitatively the free amino acid content of white blood cells from 4 normal subjects and 5 patients with chronic myeloid leukemia.

MATERIALS AND METHODS

Isolation of Leukocytes.—Blood from healthy adult donors in 20 to 25 ml. samples was drawn and mixed with heparin. The blood was centrifuged to constant cell volume and the plasma was withdrawn. The white cell layer was drawn out with minimum contamination with red cells. This layer was suspended in 2 ml. of plasma and the suspension recentrifuged in a narrow conical centrifuge tube. The white cell layer was collected again as before but with fewer erythrocytes. The cells were then briefly shaken (1 to 2 minutes) with 2 ml. of a 0.2 per cent aqueous solution of saponin whereby the red cells were lysed. Finally, the suspension was centrifuged thoroughly and the supernatant drained off.

The white cell deposit at the bottom was dispersed in twice the volume of water; 6 volumes of acetone was added, the suspension well agitated, and allowed to stand for 15 to 20 minutes. The supernatant after centrifugation was used for amino acid analysis.

In the leukemia cases where the white cell count was high it was sufficient to start with 4 to 5 ml. samples of blood.

Amino Acid Analysis.—The amino acid analyses were carried out as in the case of erythrocytes[5] except that smaller volumes of protein-free extracts were used. The volume of extract used for chromatography ranged from 5 to 60 c.mm., in terms of packed cell volume, depending on the concentration of amino acids.

From the Department of Biochemistry, Medical College, Trivandrum, India.
Received for publication Dec. 3, 1958.

RESULTS

The following ninhydrin-reacting compounds were identified on chromatograms: alanine, aspartic acid, glutamic acid, glutamine, glycine, serine, taurine, tyrosine, valine, leucine, isoleucine, and glutathione. These spots were obtained in extracts of both normal and abnormal leukocytes.

The concentration of the free amino acids in white cells (mean and standard deviation) from 4 normal subjects and 5 cases of chronic myeloid leukemia are given in Table I. It can be seen that the leukemic cells, as compared to normal ones, are richer in respect to the free amino acids, although there is considerable intragroup variation. The estimation of serine in leukemic cells was not attempted, however, since the proportion of serine to glycine was small. Hence, when a large volume of extract was used, the intense glycine spot overlapped with that of serine, whereas if smaller volumes were used, the serine spot was not of sufficient intensity.

TABLE I. CONCENTRATION OF FREE AMINO ACIDS IN LEUKOCYTES

	CONCENTRATION IN MG./100 ML. PACKED LEUKOCYTES			
	NORMAL		LEUKEMIA	
AMINO ACID	MEAN	S.D.*	MEAN	S.D.
Alanine	7.2	3.1	13.8	4.9
Aspartic acid	7.6	2.7	15.6	7.3
Glutamine	4.8	0.8	19.7	8.8
Glutamic acid	23.0	12.8	70.3	12.2
Glycine	3.7	2.3	15.3	6.8
Valine	2.2	0.8	5.4	3.0
Leucine	1.4	0.6	5.5	4.5
Isoleucine	1.0	0.4	3.1	2.4
Taurine	25.8	12.4	37.1	21.7
Serine	5.0	3.0	---	---

*Standard deviation.

Among the various amino acids, the proportion of glutamic acid and taurine is found to be conspicuously high in both normal and abnormal leukocytes. This finding is essentially in agreement with that of Nour-Eldin and Wilkinson.[4] Data on the concentrations of the other individual amino acids reported here are not available in literature.

DISCUSSION

The leukocytes present a characteristic amino acid pattern which is quite distinct from that of plasma[6] or erythrocytes[5] in several respects. The levels of alanine, aspartic acid, glycine, serine, and particularly taurine and glutamic acid are higher; the concentrations of valine, leucine, and isoleucine are of the same order; and the glutamine levels, while comparable to that of normal erythrocytes, are appreciably lower than for plasma. The most noteworthy feature, however, is the presence of taurine in very high concentration in the white cells.

We find a number of amino acids occurring in normal leukocytes in much higher concentrations than in normal plasma. The process of amino acid accumulation by cells like erythrocytes and Ehrlich ascites cells has been

extensively studied.[7] In the case of erythrocytes, amino acid accumulation is unaffected by anaerobiosis or the presence of cyanide or dinitrophenol; these findings point to the likelihood of glycolysis serving as the source of transport energy. As to whether glycolysis serves a similar function in the amino acid accumulation by leukocytes does not seem to be known. However, it is noteworthy that leukocytes which are known to be more active than erythrocytes in glycolysis[8] have been found (vide supra) to maintain higher concentrations of several amino acids than in the case of erythrocytes.

The tendency on the part of leukocytes in leukemia to accumulate several amino acids above the normal limits may be looked upon as an abnormality in the metabolism of these cells which may be either a primary aspect of the pathology of these cells or a secondary consequence. In favor of the first possibility it may be cited that rapidly growing and neoplastic cells accumulate unusually large amounts of amino acids. Further, in the case of erythrocytes in anemia, an elevation in the potassium and amino acid levels and an enhancement in the rate of glycolysis have been observed.[9]

SUMMARY

A study of the free amino acids of leukocytes from normal and leukemic subjects has been made. The presence of alanine, aspartic acid, glutamine, glutamic acid, glycine, valine, leucine, isoleucine, taurine, serine, and tyrosine has been demonstrated, and the concentrations of the first 9 estimated. In chronic myeloid leukemia the white cell concentrations of these amino acids is raised.

I wish to thank Dr. M. Sukumaran for his keen interest and the Principal, Medical College, Trivandrum, for facilities kindly provided for the work.

REFERENCES

1. Okada, S., and Hayashi, T.: Studies on the Amino Nitrogen Content of Blood, J. Biol. Chem. **51**: 121-133, 1922.
2. Sandiford, K., Boothby, W. M., and Griffin, H. Z.: The Amino Acid Nitrogen in the Blood and Its Possible Relation to the Elevation of the Metabolism in Myelogenous Leucemia, J. Biol. Chem. **55**: xxiii, 1923.
3. Schmidt, E. G.: The Amino Acid Content of the Blood in Health and in Disease, Arch. Int. Med. **44**: 351-361, 1929.
4. Nour-Eldin, F., and Wilkirson, J. F.: Amino Acid Content of White Blood Cells in Human Leukaemias, Brit. J. Haemat. **1**: 358-366, 1955.
5. Iyer, G. Y. N.: Paper Chromatographic Study of Free Amino Acids in Normal and Anaemic Erythrocytes, Indian J. M. Res. **45**: 213-216, 1957.
6. Iyer, G. Y. N.: Estimation of Free Amino Acids in Normal Human Plasma by Paper Chromatography, Indian J. M. Res. **43**: 189-193, 1955.
7. Christensen, H. N.: Mode of Transport of Amino Acids Into Cells, *in* A Symposium on Amino Acid Metabolism, Baltimore, 1955, Johns Hopkins Press, pp. 63-105.
8. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry, Interpretations, Baltimore, 1946, Williams & Wilkins Company, vol. I, p. 161.
9. Iyer, G. Y. N.: Free Amino Acid Levels in Normal and Anemic Erythrocytes in Relation to Their Potassium Content and Rate of Glycolysis, Arch. Biochem. **74**: 24-27, 1958.

THE HEMOLYTIC EFFECT OF PRIMAQUINE

X. Aldolase and Glyceraldehyde-3-Phosphate Dehydrogenase Activity in Primaquine-Sensitive Erythrocytes

Stanley L. Schrier, M.D.,* Robert W. Kellermeyer, M.D.,** Paul E. Carson, M.D.,*** Charles E. Ickes, M.T.,**** and Alf S. Alving, M.D.***
Chicago, Ill.

WE WISH to present results of studies on the glycolytic pathway in primaquine-sensitive erythrocytes, because these cells are deficient in an alternative pathway of glucose metabolism. This genetically determined susceptibility to drug-induced hemolytic anemia occurs in approximately 10 per cent of otherwise healthy American Negroes.[1] The distribution of this trait is widespread and other ethnic groups, including Italians and dark-skinned Sephardic Jews, are frequently affected.[2-4] The expanding list of agents which can precipitate this hemolytic anemia includes primaquine,[5] sulfanilamide,[6] nitrofurantoin (Furadantin),[7] furazolidone (Furoxone),[8] and fava beans.[2] Because the original investigations were done with primaquine,[5] we have referred to this inborn error of metabolism as primaquine-sensitive hemolytic anemia. Primaquine-sensitive erythrocytes are normal morphologically and no abnormal erythrocytic antibodies have been detected.[9] The erythrocytes, however, are characterized by at least 5 intrinsic biochemical abnormalities (Table I).[10-15]

Primaquine-sensitive erythrocytes, deficient in glucose-6-phosphate dehydrogenase (G-6-PD) activity,[13] the enzyme catalyzing the initial oxidative reaction in the hexosemonophosphate shunt (Reaction 1) (Fig. 1), would metabolize less glucose through this pathway.[16] Nevertheless, glucose utilization is normal in sensitive erythrocytes,[10, 17] indicating that more glucose may be channeled through the Embden-Meyerhof pathway.

Glyceraldehyde phosphate dehydrogenase (GAPD) catalyzes a rate limiting reaction in the Embden-Meyerhof pathway (Reaction 4) (Fig. 1).[18] Aldolase provides substrate for GAPD by catalyzing the reversible splitting of

From the University of Chicago Army Malaria Research Unit, Chicago, Ill.

This work was supported (in major part) by the Research and Development Division, Office of the Surgeon General, Department of the Army, under Contract No. DA-49-007-MD-566 with the Department of Medicine, University of Chicago, Chicago, Ill. Additional support was obtained from Winthrop Laboratories, Inc., New York, N. Y.; Parke, Davis & Company, Detroit, Mich.; and the Douglas Smith Foundation, University of Chicago, Chicago, Ill.

The investigations reported in this paper would not have been possible except for the cooperation of the inmates and the administrative staff of Stateville Penitentiary, Joliet, Ill.

Received for publication Nov. 17, 1958.

*Department of Medicine, University of Chicago, Chicago, Ill., Formerly S. A. Surgeon (R), United States Public Health Service.

**S. A. Surgeon (R), United States Public Health Service.

***Department of Medicine, University of Chicago, Chicago, Ill.

****Medical Technician.

TABLE I. BIOCHEMICAL ABNORMALITIES

1. Low reduced glutathione (GSH) but normal oxidized glutathione
2. Unstable GSH* which falls in vivo when hemolytic drug is administered, and falls in vitro when red blood cells are incubated with APH
3. Deficiency of glucose-6-phosphate dehydrogenase activity
4. Increase in glutathione reductase activity
5. Increase in aldolase activity

*Key to abbreviations used in tables and text: ADP, adenosinediphosphate; ATP, adenosinetriphosphate; APH, acetylphenylhydrazine; DHAP, dihydroxyacetone phosphate; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; FDP, fructose-1, 6-diphosphate; GA-3P, glyceraldehyde-3-phosphate; GAPD, glyceraldehyde phosphate dehydrogenase; G-6-P, glucose-6-phosphate; G-6-PD, glucose-6-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione; Pi, inorganic phosphate; 3-PGA, 3-phosphoglycerate; 6-PG, 6-phosphogluconate; 6-PGD, 6-phosphogluconic dehydrogenase; PGK, phosphoglyceric kinase; P_5P, pentose phosphate; TA, transaldolase; TI, triosephosphate isomerase; TK, transketolase; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.

FDP into two moles of triosephosphate (Reaction 3). These enzymes were assayed in hemolyzates of primaquine-sensitive and nonsensitive erythrocytes.

The following reactions will be referred to in these investigations:

$$(1)\ \text{G-6-P} + \text{TPN}^+ \xrightarrow{\text{G-6-PD}} \text{6-PG} + \text{TPNH} + \text{H}^+$$

$$(2)\ \text{GSSG} + \text{TPNH} + \text{H}^+ \xrightarrow[\text{reductase}]{\text{glutathione}} 2\ \text{GSH} + \text{TPN}^+$$

$$(3)\ \text{FDP} \underset{}{\overset{\text{aldolase}}{\rightleftharpoons}} \text{GA-3-P} + \text{DHAP}$$

$$(4)\ \text{GA-3P} + \text{DPN}^+ + \text{ADP} + \text{Pi} \xrightarrow[\text{PGK}]{\text{GAPD}} \text{3-PGA} + \text{DPNH} + \text{ATP}$$

$$(5)\ \text{GA-3P} \underset{\text{isomerase}}{\overset{\text{triosephosphate}}{\rightleftharpoons}} \text{DHAP}$$

$$(6)\ \text{DHAP} + \text{DPNH} + \text{H}^+ \underset{\text{dehydrogenase}}{\overset{\text{alpha-glycerophosphate}}{\rightleftharpoons}} \text{alpha-glycerophosphate} + \text{DPN}^+$$

MATERIALS AND METHODS

Subjects were adult male volunteers. Those designated as "primaquine-sensitive" demonstrated acute intravascular hemolysis on a daily test dose of 30 mg. of primaquine base for at least one week. No sensitive individual was studied who had undergone hemolysis within the previous 4 months. Nonsensitive individuals did not undergo hemolysis with this treatment or with an eightfold increase in primaquine. No individual studied was iron deficient.

Barium fructose diphosphate (Schwarz Laboratories, Inc.) was converted to the sodium salt and solutions were brought to pH 7.2 with NaOH. Approximately 8 per cent of this material consisted of hexosemonophosphates. GSH was obtained from Schwarz Laboratories and its purity was checked as previously described.[11] DPN* was 93 per cent pure as determined enzymatically and contained no TPN by specific assay. DPNH* was assayed both spectrophotometrically and enzymatically before use. Tris-(hydroxymethyl)aminomethane buffer (Sigma 121) was adjusted with hydrochloric acid to pH 7.4, 7.7, and 8.4. Dihydroxyacetone phosphate (California Foundation for

*Obtained from the Sigma Chemical Company, St. Louis, Mo.

Biochemical Research), prepared as directed by the manufacturer, was 92 to 96 per cent pure by assay with alpha-glycerophosphate dehydrogenase.* Aldolase and glyceraldehyde phosphate dehydrogenase were obtained from the Worthington Biochemical Corporation. The glyceraldehyde phosphate dehydrogenase contained significant amounts of triosephosphate isomerase and very small amounts of aldolase.

I. Preparation of Hemolyzates.—

A. Preparation of standard hemolyzate: Standard hemolyzate was prepared as described previously.[14] The presence of hexosemonophosphates in our FDP made it necessary to remove any remaining coenzymes so that interfering reactions would not occur. Therefore, 15 mg. of acid-washed Norit A per milliliter of dialyzed hemolyzate was added and the mixture was shaken for 10 minutes. Norit treatment removed all the remaining coenzymes (to less than 10^{-7} M by enzymatic assay) and was not deleterious to the enzymes under study.

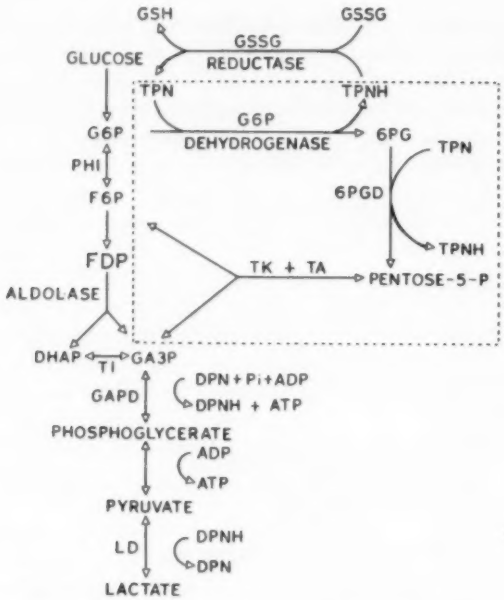


Fig. 1.—This represents an abbreviated schematic outline of glucose metabolism in the erythrocyte. The dotted line encloses the reactions of the hexosemonophosphate shunt.

B. Preparation of hemoglobin-free hemolyzate: We have modified the method of Huennekens and associates[19] by adding more ethanol and chloroform in order to remove interfering glycolytic enzymes as well as hemoglobin. Fortunately, glyceraldehyde phosphate dehydrogenase is quite resistant to this treatment and the hemoglobin-free hemolyzate can be used as a source of this enzyme.

Freshly drawn heparinized venous blood was immediately centrifuged in an International PR-2 refrigerated centrifuge at 1,560 g (4° C.) for 15 minutes. The plasma and buffy coat were removed and the erythrocytes were washed once in cold (10° C.) 0.145 M NaCl. One volume of packed erythrocytes was hemolyzed by freezing and thawing 3 times. Then one volume of cold 0.145 M NaCl and 4 volumes of cold distilled water were added, and the mixture was gently swirled. In rapid succession 1.6 volumes of

ethanol and 1 volume of chloroform (both redistilled and stored at −20° C.) were added, the mixture was shaken vigorously for 1 minute, then centrifuged immediately for 10 minutes at 1,500 g (2° C.). The supernatant solution was decanted through a medium sintered glass filter. Two hundred milliliters of clear filtrate were placed into a Visking cellulose bag (pore size 24 Å., filled diameter 1⅝ in.) and dialyzed against 10,000 ml. of M/15 phosphate buffer, pH 7.4, with constant motion for 16 to 18 hours at 4° C. The dialyzate was lyophilized and stored at −20° C. This hemolgobin-free hemolyzate (less than 0.01 Gm. per cent hemoglobin) contained very active GAPD and triosephosphate isomerase, insignificant amounts of both aldolase and glutathione reductase, and no G-6-P, 6-P-G, FDP, GA-3P, DHAP, G-6-PD, 6-PGD, or alpha-glycerophosphate dehydrogenase by specific enzymatic measurements. Hemoglobin-free hemolyzate prepared from normal and primaquine-sensitive erythrocytes could then be assayed for GAPD activity, since interfering enzymes and hemoglobin had been removed.

The lyophilized material was also concentrated (so that 1 ml. of aqueous solution contained the equivalent of 2.5 ml. of dialyzed hemolyzate) and used as a source of GAPD in the assay for aldolase activity. (See the following section.)

II. Methods for Assaying Aldolase Activity.—Our method is based on that of Warburg and Christian,[20] which measures the reduction of DPN spectrophotometrically at 340 mμ. Both GA-3P and DHAP formed by the action of aldolase on FDP (Reaction 3) will result in the reduction of DPN in the presence of triosephosphate isomerase (Reaction 5). The addition of excess arsenate to Reaction 4 makes it irreversible, prevents triosephosphate from recycling through the glycolytic pathway, and makes DPNH a valid end product to measure. Because standard Norit-treated hemolyzate contains hemoglobin and interfering enzymes, we divided the aldolase assay into two steps.

A. Reaction step (corresponds to Reaction 3): Reaction mixtures containing 1.0 ml. of tris 0.5 M pH 7.4, 0.5 ml. FDP 0.065 M, and aliquots of stroma-free, Norit-treated hemolyzate were brought to final volume of 7.0 ml. with distilled water and incubated in a water bath at 37° C. for the required period of time. We discovered no cationic requirements for this reaction and the addition of Versene (0.005 M) caused no significant change. The reaction was stopped by adding 5.0 ml. of 4.5 per cent metaphosphate solution; the resulting mixture was swirled thoroughly for 5 minutes and decanted through Whatman No. 2 filter paper. Eight milliliters of filtrate was transferred to a 10 ml. volumetric flask, neutralized to pH 7.2 with 1.0 N NaOH, and brought to 10 ml. Blanks contained all reagents except that the metaphosphate was added before the hemolyzate.

B. Assay step (corresponds to Reactions 4 and 5): Both GA-3P and DHAP formed by the action of hemolyzate aldolase on FDP were estimated in the neutralized protein-free filtrates as follows:

Assays containing 1.0 ml. tris buffer 0.5 M pH 8.4, 0.5 ml. GSH 0.1 M, 0.5 ml. sodium arsenate 0.088 M 1.0 ml. DPN 17.6×10^{-3} M, 1 ml. of concentrated hemoglobin-free hemolyzate (containing GAPD and triosephosphate isomerase, but no DPNH oxidases or phosphatases as determined by specific enzymic assays), and 1.0 ml. neutralized filtrate (Step A) were incubated at 20° C. Metaphosphate slowed the reaction time so that 90 minutes of incubation was required for maximum reduction of DPN. At that time aliquots of these assays were transferred to 1 cm. light path cuvettes and read against the blank (which contained all reagents) at 340 mμ in the Beckman DU spectrophotometer.

Experiments in which DHAP was added to either the reaction or the assay yielded recoveries of 90 to 95 per cent. Commercial preparations of GAPD and triosephosphate isomerase used in the assay step gave similar results. As suggested by Baranowski and Niederland,[21] another method for determining aldolase activity was used to check our results. This method takes advantage of the equilibrium of triosephosphate isomerase, which converts 95 per cent of the triosephosphate to DHAP. DHAP was estimated by the oxidation of DPNH in the presence of alpha-glycerophosphate dehydrogenase (Reaction 6). Studies with this method gave entirely comparable results.

Duplicate determinations were within 4 per cent of each other. The variation of daily aldolase determinations in nonsensitive individuals was within the error of the method. Aldolase activity was referred to the hemoglobin content of the reaction hemolyzate.

III. Method for Assaying Glyceraldehyde Phosphate Dehydrogenase.—This method is also based on that of Warburg and Christian.[20] Commercial aldolase, free of GAPD, was added to FDP to provide a source of GA-3P. Then hemoglobin-free hemolyzate was added and the reduction of DPN was measured spectrophotometrically at 340 mμ (Reaction 4).

Reaction mixtures containing 1.0 ml. tris 0.5 M pH 7.7, 0.5 ml. GSH 0.1 M, 0.5 ml. sodium arsenate 0.088 M, 0.5 ml. aldolase 1,400 units, 0.5 ml. H_2O, and 0.5 ml. FDP 7×10^{-3} M were "preincubated" for 15 minutes at 20° C. to provide a source of GA-3P. Then 1.0 ml. of DPN 17.6×10^{-3} M and 0.5 ml. of hemoglobin-free hemolyzate (diluted with water to contain the equivalent of 0.25 ml. dialyzed hemolyzate) was added. Aliquots were immediately transferred to 1 cm. light path cuvettes and read at 340 mμ in the Beckman DU spectrophotometer against a blank which contained all reagents except FDP. The activity of GAPD was referred to in the protein content of hemoglobin-free hemolyzate which was estimated spectrophotometrically by absorption at 260 and 280 mμ.[22] TPN cannot serve as coenzyme for GAPD in human hemolyzates.

RESULTS

A. Assays of Aldolase and Glyceraldehyde Phosphate Dehydrogenase in Hemolyzates.—Assays of aldolase and glyceraldehyde phosphate dehydrogenase were performed on hemolyzates from primaquine-sensitive and nonsensitive volunteers. Results are summarized in Fig. 2. Aldolase activity in hemolyzates from primaquine-sensitive erythrocytes was consistently increased and there was no overlap between the two groups. In contrast, no difference in glyceraldehyde phosphate dehydrogenase activity was demonstrated.

B. The Relationship of Reaction Time and Hemolyzate Concentration to Aldolase Activity.—A typical study comparing the relationship of reaction time and hemolyzate concentration to aldolase activity in hemolyzates from primaquine-sensitive and nonsensitive men is shown in Fig. 3. Seventeen primaquine-sensitive individuals and 14 nonsensitive individuals have been tested, and the aldolase activity was consistently increased by approximately 30 per cent in the sensitive individuals. The increase was independent of considerable variation in temperature (20° to 60° C.), pH (6.7 to 8.4), substrate concentration (0.0001 M to 0.01 M), and conditions of aerobiosis and anaerobiosis.

C. Effect of Primaquine Administration on Aldolase Activity.—To determine the effect of drug administration on aldolase activity in hemolyzates from nonsensitive and primaquine-sensitive men, assays were performed on 7 individuals (4 nonsensitive and 3 sensitive) before, during, and after primaquine administration. No change in aldolase activity was observed during drug administration to normal individuals. The increase in aldolase activity in primaquine-sensitive erythrocytes persisted through the period of drug administration (Fig. 4). No change in GAPD activity was detected by our method during primaquine administration in either the primaquine-sensitive or nonsensitive subject.

D. Effect of "Primaquine Plasma" on Aldolase Activity In Vitro.—A nonsensitive individual was fed 240 mg. primaquine base in 8 divided doses during a 24-hour period. Aliquots of this "primaquine plasma" and drug-free plasma were added to the reaction step in aldolase determinations. Table II indicates that neither normal nor "primaquine plasma" altered aldolase activity in hemolyzates of either nonsensitive or primaquine-sensitive erythrocytes.

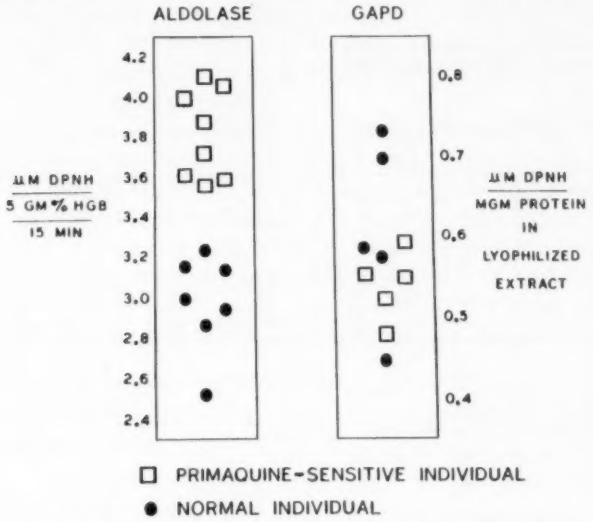


Fig. 2.—Activities of aldolase and glyceraldehyde phosphate dehydrogenase as determined, respectively, in hemolyzates and hemoglobin-free extracts.

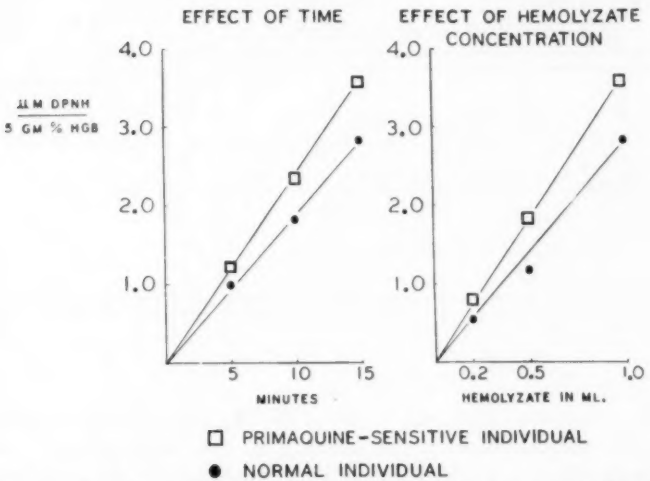


Fig. 3.—The relationship of reaction time and hemolyzate concentration to aldolase activity in hemolyzates from primaquine-sensitive and nonsensitive men.

TABLE II. IN VITRO EFFECT OF PRIMAQUINE PLASMA ON ALDOLASE ACTIVITY

EXPERIMENTAL CONDITIONS	ALDOLASE ACTIVITY (μM TRIOSEPHOSPHATE/5 GM. % HGB)
Primaquine-sensitive erythrocytes	2.64
Primaquine-sensitive erythrocytes plus normal plasma	2.82
Primaquine-sensitive erythrocytes plus primaquine plasma	2.60
Normal erythrocytes	2.40
Normal erythrocytes plus normal plasma	2.34
Normal erythrocytes plus primaquine plasma	2.36
Normal plasma	0.18
Primaquine plasma	0.11

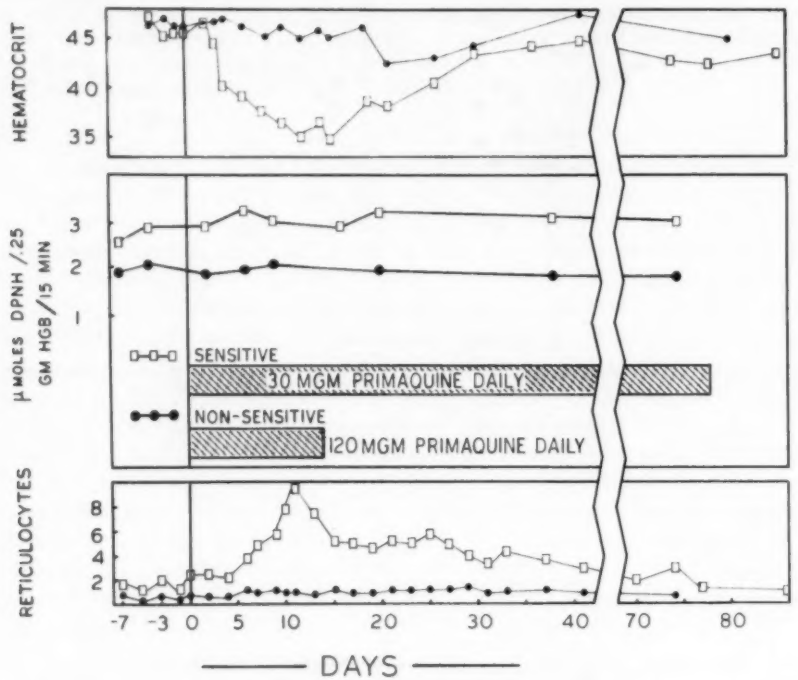


Fig. 4.—Aldolase activity during primaquine administration to sensitive and a nonsensitive man. The aldolase activity of a nonsensitive man not taking drug was similar to that of the nonsensitive man taking drug, as shown in the figure.

DISCUSSION

Under in vitro conditions which approximate the physiologic state, normal human erythrocytes metabolize 10 to 20 per cent of their total glucose uptake via the oxidative limb of the hexosemonophosphate shunt.[23] Primaquine-sensitive erythrocytes deficient in G-6-PD are deprived of normal use of the

hexosemonophosphate shunt; nevertheless, their total glucose utilization is normal. We have investigated mechanisms by which more glucose may be channeled through the Embden-Meyerhof pathway.

The presence in human erythrocytes of comparatively large amounts of FDP (1×10^{-4} moles per liter erythrocytes; substrate for aldolase)[24] and the absence of triosephosphate, the end product of aldolase and substrate for GAPD, suggests that the level of aldolase activity may limit utilization of the Embden-Meyerhof pathway by erythrocytes. Increased aldolase activity in primaquine-sensitive erythrocytes may aid in channeling more glucose through the Embden-Meyerhof pathway.

Larizza and associates,[3] using 0.05 ml. amounts of hemolyzate as a source of aldolase activity in a colorimetric method,[25] found that patients susceptible to fava bean-induced hemolysis had mean values of aldolase activity which were higher than normal. Because of the large overlap between the two groups, they did not consider the difference to be significant. Our modifications of two enzymatic methods, using larger amounts of hemolyzate, assay aldolase activity more accurately and quantitatively. We found distinct differences between the two groups of men studied, without overlap in 31 subjects.

SUMMARY

1. We have adapted the methods of Warburg and Christian and Baranowski and Niederland for the quantitative assay of aldolase activity in human hemolyzates. Glyceraldehyde phosphate dehydrogenase was estimated on hemoglobin-free hemolyzates.

2. Primaquine-sensitive erythrocytes have approximately 30 per cent more aldolase activity than normal. In contrast, no difference in GAPD activity was detected by our method.

3. Neither aldolase activity nor GAPD activity changes significantly during primaquine administration.

4. Increased aldolase activity may aid primaquine-sensitive erythrocytes to metabolize more glucose via the Embden-Meyerhof pathway.

REFERENCES

1. Alving, A. S., Kellermeyer, R. W., Tarlov, A., Schrier, S., and Carson, P.: Some Genetic Aspects of Primaquine-sensitive Hemolytic Anemia, Ann. Int. Med. **49**: 240-248, 1958.
2. Szeinberg, A., Sheba, C., and Adams, A.: Enzymatic Abnormality in Erythrocytes of a Population Sensitive to Vicia Faba or Haemolytic Anaemia Induced by Drugs, Nature **181**: 1256, 1958.
3. Larizza, P., Bruetti, P., Grignana, F., and Ventura, S.: L'individualita bio-enzimatica dell'eritrocito fabico, Haematologica **43**: 205-250, 1958.
4. Sansone, G., and Segal, G.: Nuovi aspetti dell'alterato biochimismo degli eritrociti de favice: assenze pressoche completa della glucoso-6-deidrogenasi, Boll. Soc. ital. biol. sper. **34**: 327-329, 1958.
5. Hockwald, R. S., Arnold, J., Clayman, C. B., and Alving, A. S.: Status of Primaquine. 4. Toxicity of Primaquine in Negroes, J. A. M. A. **149**: 1568-1570, 1952.
6. Dern, R. J., Beutler, E., and Alving, A. S.: The Hemolytic Effect of Primaquine. V. Primaquine Sensitivity as a Manifestation of a Multiple Drug Sensitivity, J. Lab. & Clin. Med. **45**: 30-39, 1955.

7. Kimbro, E. L., Jr., Sachs, M., and Torbert, J. V.: Mechanism of the Hemolytic Anemia Induced by Nitrofurantoin (Furadantin), Bull. Johns Hopkins Hosp. **101**: 245-274, 1957.
8. Kellermeyer, R. W., Tarlov, A. R., Schrier, S. L., and Alving, A. S.: The Hemolytic Effect of Commonly Used Drugs on Erythrocytes Deficient in Glucose-6-Phosphate Dehydrogenase, J. LAB. & CLIN. MED. **52**: 827-828, 1958.
9. Beutler, E., Dern, R. J., and Alving, A. S.: The Hemolytic Effect of Primaquine. III. A Study of Primaquine-Sensitive Erythrocytes, J. LAB. & CLIN. MED. **44**: 177-184, 1954.
10. Beutler, E., Dern, R. J., Flanagan, C. L., and Alving, A. S.: The Hemolytic Effect of Primaquine. VII. Biochemical Studies of Drug-Sensitive Erythrocytes, J. LAB. & CLIN. MED. **45**: 286-295, 1955.
11. Flanagan, C. L., Schrier, S. L., Carson, P. E., and Alving, A. S.: The Hemolytic Effect of Primaquine. VIII. The Effect of Drug Administration on Parameters of Primaquine Sensitivity, J. LAB. & CLIN. MED. **51**: 600-608, 1958.
12. Beutler, E.: The Glutathione Instability of Drug-Sensitive Red Cells. A New Method for the In Vitro Detection of Drug Sensitivity, J. LAB.. & CLIN. MED. **49**: 84-95, 1957.
13. Carson, P., Flanagan, C. L., Ickes, C. E., and Alving, A. S.: Enzymatic Deficiency in Primaquine-Sensitive Erythrocytes, Science **124**: 484-485, 1956.
14. Schrier, S. L., Kellermeyer, R. W., Carson, P. E., Ickes, C. E., and Alving, A. S.: The Hemolytic Effect of Primaquine. IX. Enzymatic Abnormalities in Primaquine-Sensitive Erythrocytes, J. LAB. & CLIN. MED. **52**: 109-117, 1958.
15. Schrier, S. L., and Kellermeyer, R. W.: Compensatory Mechanisms in Primaquine-Sensitive Erythrocytes, J. Clin. Invest. **37**: 928, 1958.
16. Johnson, A. B., and Marks, P. A.: Glucose Metabolism and Oxygen Consumption in Normal and Glucose-6-Phosphate Dehydrogenase Deficient Human Erythrocytes, Clin. Res. (Abst.) **6**: 187-188, 1958.
17. Schrier, S. L.: Unpublished Observations.
18. Krebs, H. A.: Control of Metabolic Processes, Endeavor **16**: 125-132, 1957.
19. Huennekens, F. M., Caffrey, R. W., Basford, R. E., and Gabrio, B. W.: Erythrocyte Metabolism. IV. Isolation and Properties of Methemoglobin Reductase, J. Biol. Chem. **227**: 261-272, 1957.
20. Warburg, O., and Christian, W.: Isolierung und Krystallisation des Gurungs ferment: Zymohexase, Biochem. Ztschr. **314**: 149-176, 1943.
21. Baranowski, T., and Niederland, T. R.: Aldolase Activity of Myogen A, J. Biol. Chem. **180**: 543-551, 1949.
22. Nielands, J. B., and Stumpf, P. K.: Outlines of Enzyme Chemistry, New York, 1955, John Wiley & Sons, Inc., pp. 24-25.
23. Murphy, J. R.: Demonstration of Aerobic Oxidative Glucose Metabolism in the Erythrocytes, J. LAB. & CLIN. MED. **50**: 936-937, 1957.
24. Bartlett, G. R., Savage, E., Hughes, L., and Marlow, A. A.: Carbohydrate Intermediates and Related Cofactors in the Human Erythrocyte, J. Appl. Physiol. **6**: 51-56, 1953-54.
25. Bruns, F.: Bestimmung und Eigenschaften der Serumaldolase, Biochem. Ztschr. **325**: 156-162, 1954.

ANTIGENICITY OF HUMAN VASCULAR ENDOTHELIUM: LACK OF RELATIONSHIP TO THE PATHOGENESIS OF VASCULITIS

SERGIO PIOMELLI, M.D.,* MARIO STEFANINI, M.D.,** AND ROSE H. MELE, B.S.***
BOSTON, MASS.

MECHANISMS of autoimmunization are frequently considered in the pathogenesis of some disorders involving the formed elements of the blood. The recognition of an autoimmune mechanism in the etiology of a blood disorder is usually based on the fulfillment of 4 basic postulates[1]: (1) the demonstration of the antigenicity of the cell involved; (2) the presence in the patient's blood of substances reacting with normal blood cells in vitro; (3) the shortened survival time of normal cells injected into the patient's circulation; and (4) the possibility of reproducing the syndrome in a normal individual by means of the passive transfer of a sufficient amount of the patient's plasma or serum. On the basis of these concepts, certain types of idiopathic anemia, leukopenia, thrombocytopenia, and of multiple cytopenia are classified as autoimmune disorders.

More recently, an autoimmune mechanism has also been postulated for anaphylactoid purpura, periarteritis nodosa, and other forms of vasculitis. Some indirect support of this theory has come from experiments suggesting the heteroantigenicity of the vascular endothelium. Vascular purpura has been produced with the injection of an antiserum into the animal donating the vascular antigen.[2, 3] A precipitin reaction has been described between sera of patients with anaphylactoid purpura or periarteritis nodosa and extracts of human aorta.[4] The specificity of this reaction seems supported by additional work with the antihuman globulin consumption test.[5] Finally, histologic lesions similar to those seen in human vasculitis have been produced with the subcutaneous injection of serum from these patients into guinea pigs.[6] Most of these experiments, however, appear poorly controlled. There are not even clear-cut experiments to date demonstrating the antigenicity of human vascular endothelium or of any of the constituents of the human vascular wall.

From the Joseph Stanton Memorial Laboratories, Saint Elizabeth's Hospital, and the Department of Medicine, Tufts University School of Medicine, Boston, Mass.

Parts of this work were presented at the Annual Meeting of the American Society for Clinical Investigation, Atlantic City, N. J., May 3, 1954, at the Annual Meeting of the American Society of Immunologists, Philadelphia, Pa., April 14, 1958, and at the 7th Congress of the International Society of Hematology, Sept. 13, 1958.

Received for publication Dec. 8, 1958.

*Fulbright Scholar in Medical Sciences; on leave of absence from the Department of Medical Pathology, University of Naples.

**Director of Research and Hematologist, Joseph Stanton Memorial Laboratories, Saint Elizabeth's Hospital; Professor of Medicine, Tufts University School of Medicine, Boston, Mass.

***Head Research Technician, Joseph Stanton Memorial Laboratories, Saint Elizabeth's Hospital.

With these considerations in mind, the purpose of this paper is threefold: to establish the presence of antigenic substances in the human vessel wall and its constituents; to characterize their organ, tissue, and species specificity; and to study further the nature of the precipitin reaction which has been described in the serum of patients with anaphylactoid purpura and periarteritis nodosa.

MATERIALS AND METHODS

Materials.—New Zealand albino rabbits (of average weight between 1,500 and 2,000 grams) were used for preparation of the antihuman vessel antisera.

a. Collection of constituents of the vascular wall: Human aortas, grossly free of sclerotic changes, were collected within two hours of death and washed many times in cold saline. Intima, media, and adventitia were each isolated from the aorta as well as possible by careful scraping. The separation of the intima from aorta in human beings appeared simple, as compared to the difficulty encountered in animals. The clean tissue was then frozen at −20° C. until used. Human placenta was collected also within two hours of delivery. The main vessels were carefully isolated, cut longitudinally, washed in cold saline, and frozen at −20° C. Human kidneys, livers, and spleens were similarly collected within two hours of death, freed of capsule, fat, and main vessels, finely minced with scissors, washed many times in cold saline, and kept frozen at −20° C. The intima from the aorta of several species (dog, rabbit, guinea pig, calf, and sheep) was isolated as described elsewhere.[7]

b. Preparation of water-soluble organ extracts: The frozen tissues were thawed slowly, washed twice in distilled water to remove as much blood as possible, then homogenized with distilled water in a Waring Blendor and kept at 4° C. Homogenization was carried out at periods of 2 minutes each at 1-hour intervals for 5 times, to avoid changes in temperature. Finally, the material was transferred to a Potter glass homogenizer, kept in a melting ice beaker and treated for 3 minutes. After centrifugation at 40,000 g for 10 minutes at 4° C., the supernatant fluid was separated and lyophilized. With this technique water-soluble extracts were prepared from intima, media, and adventitia of human aorta and from placental vessels. The protein content ranged from 44.4 to 58.8 per cent of lyophilized material. Water-soluble extracts were also obtained from liver, kidney, and splenic tissues.

c. Preparation of total aorta endothelial homogenates: The isolated intimas from human aortas were washed many times in saline. They were then homogenized as described in the preceding paragraph, first in a Waring Blendor and then in a Potter glass homogenizer in cold saline (10 per cent w/v concentration). The extract was kept frozen at −20° C. until used.

d. Preparation of platelet suspensions: This was carried out as previously described from this laboratory.[8] Final suspensions contained 300,000 platelets per cubic millimeter.

e. Preparation of antisera against water-soluble extracts of human vessels (Table I): The lyophilized antigens were dissolved in saline and, after incorporation in alumina gel, were injected intramuscularly in 4 groups, each composed of 2 rabbits. Each animal received a total of 9 injections at 2-day intervals, each injection corresponding to 10 mg. of protein antigen in 2 ml. of alumina gel. A second course of 5 injections was given to each animal 50 days later. Sera were collected 8 to 10 days after the last immunizing injection from the medial artery of the ear. The following sera were produced with this technique, using water-soluble antigens (Table I): antihuman intima (rabbit) serum (Anti-HI); antihuman adventitia (rabbit) serum (Anti-HA); antihuman media (rabbit) serum (Anti-HM); antihuman placental vessel (rabbit) serum (Anti-HPV).

f. Preparation of antiserum against human endothelial homogenates: The antigen was incorporated in an equal volume of alumina gel and injected intraperitoneally into 2 rabbits.

Each animal received three 5 ml. injections of the antigen at 3-day intervals. Fifty days later each animal received another course of two 6 ml. injections. Sera were collected 8 and 10 days after the last immunizing injections. The serum obtained was designated as antihuman intima (total antigen) (rabbit) serum (Anti-HE).

g. Antiserum against human platelets and human serum proteins: These were kindly supplied by Dr. Shokichi Ueno* and prepared as described by this author.[8] They were designated as antihuman platelets (rabbit) serum (Anti-HPl), and antihuman serum (rabbit) serum (Anti-HS).

h. Miscellaneous reagents: Magnesium veronal buffer (pH 7.3), prepared according to Mayer,[9] was used in all serologic experiments. One-tenth per cent gelatin was added when the buffer was used for complement fixation experiments. Commercial lyophilized guinea pig serum was used as complement. The hemolytic system for complement fixation experiments was as follows. Sheep blood was collected and preserved in Alsever's solution under sterile conditions. It was stored at 4° C. for not less than 5 days and not longer than 8 weeks. At the time of use the erythrocytes were washed in saline buffer, filtered through Whatman No. 42 paper and suspended in gelatin buffer. For 50 and 100 per cent hemolysis experiments the suspensions were standardized to contain 10^9 red cells per milliliter using the Coleman Junior spectrophotometer and 13 mm. diameter matched cuvettes. The erythrocytes were then sensitized with an equal volume of rabbit antisheep erythrocyte serum, supplying 4 minimal hemolytic units.

Methods.—

a. One hundred per cent hemolytic units complement fixation: Test tubes were incubated 1 hour at 4° C. and 1 hour at 37° C. A constant amount of complement (2 units) was used. Readings were made after 30 minutes of hemolytic action. Tests were always made using several (twofold) dilutions of antisera and several (fivefold) dilutions of the antigen. Results were expressed as the reciprocal of the maximum dilution of serum giving no hemolysis with the optimal dilution of antigen.

b. Fifty per cent hemolytic units complement fixation: A constant dilution (1:100) of antiserum was tested against several dilutions of antigen, in presence of variable amounts of complement (from 25 to 100 fifty per cent hemolytic units) in a final volume of 10 ml. and for an incubation period of 20 hours at 4° C. A hemolytic period of 60 minutes was used constantly. Readings were made in the Coleman Junior spectrophotometer, in the range of 30 to 70 per cent hemolysis, according to the original technique.[10] Complement units were calculated from the Van Krogh equation, for $1/n = 0.2$ (a constant value in our experimental conditions), and the results were expressed as 50 per cent hemolytic complement-fixing units per milliliter in the optimal antigen/antibody ratio. Slight anticomplementary activities of antigens and antisera were subtracted in calculations.

c. Titration of Forssman antibodies: This procedure was as described by Kabat,[11] in the presence of two 100 per cent hemolytic units of complement.

d. Precipitation (ring test): The precipitation test was performed in capillary tubes. Titrations were always carried out diluting the antisera twofold in buffer containing 1.5 per cent gum arabic and overlaying the antigen, diluted fivefold in buffer. Results were expressed as the highest dilution of serum giving a clear precipitation ring with the optimal dilution of antigen after one hour. (Optimal antigen/antibody ratio, although present, was not as sharp with this method as with other serologic techniques.)

e. Agar precipitation plate technique: The method used was that of Ouchterlony[13] employing a 2 mm. thickness layer of 1.5 per cent agar in saline containing 1:10,000 merthiolate. The wells were filled a second time within the first 24 hours. Results were read after 5 days of incubation at room temperature and after 10 days of incubation at 4° C.

*Professor of Legal Medicine, University of Tokyo, Japan. Formerly Visiting Fellow of the China Board of New York City at this institution.

f. Absorption of antibodies: Antigens were added to the antisera in slight excess of the optimal ratio. Optimal antigen/antibody ratio was determined according to the method of Dean and Webb.[12] The mixtures were incubated 24 hours at 4° C., then centrifuged at 40,000 g for 20 minutes at 4° C., and the supernatant fluid retested. Further additions of one-fourth the amount of antigen originally added and incubations at 4° C. for 24 hours were repeated when necessary until ring precipitation and complement fixation in the supernatant were negative.

g. Protein determinations: Protein determinations were carried out by the Markham modification of the micro-Kjeldahl technique.[14]

h. Erythrocyte agglutination test: This test was performed at 37° C. with an incubation period of 60 minutes. Serial dilutions in saline of serum were prepared. One-tenth milliliter of each serum dilution and 0.05 ml. of 1 per cent suspension of red blood cells were used in each test.

i. Localization of antibodies after tagging with fluorescein isocyanate: Human organs (kidney, liver, and spleen) were obtained at autopsy and immediately frozen in a mixture of ethanol and solid CO_2. Frozen sections were cut at 4 μ thicknesses. Localization was studied on unfixed sections as well as on sections fixed in 10 per cent formalin. The indirect technique of Watson and Coons[15] was used. In a first step, the sections were treated with antihuman vessel serum which had been previously absorbed with human serum. In a second step an antirabbit globulin goat serum* was used, tagged with fluorescein isocyanate, the unspecific staining activity of which had been removed by repeated absorptions on acetone-dry human liver powder. The specificity of the localization was controlled, and for each section an adequate series of controls was prepared treated (a) with buffered saline; (b) with buffered saline, then with fluorescent antirabbit globulin goat serum; (c) with normal rabbit serum, then with fluorescent antirabbit globulin goat serum; (d) with antidog-endothelium rabbit serum, then with fluorescent antirabbit globulin goat serum; (e) with antihuman vessel rabbit serum, then with buffered saline. When localizing activity was present, antibodies were removed from the serum by absorption on the antigen used for immunization. The serum was then retested to confirm the disappearance of specific localizing activity.

RESULTS

1. Antigenic Structure of Human Endothelium.—The injection of human endothelial extract in rabbits stimulated the production of heteroantibodies (Table I). There was considerable difference from one animal to another, titers

TABLE I. KEY TO THE ABBREVIATIONS USED TO DESIGNATE VARIOUS ANTISERA

Anti-HI	= Antihuman intima (water-soluble extract) (rabbit) serum
Anti-HA	= Antihuman adventitia (water-soluble extract) (rabbit) serum
Anti-HM	= Antihuman media (water-soluble extract) (rabbit) serum
Anti-HVP	= Antihuman placental vessels (water-soluble extract) (rabbit) serum
Anti-HE	= Antihuman endothelium (intima) (total extract) (rabbit) serum
Anti-HPl	= Antihuman platelets (rabbit) serum
Anti-HS	= Antihuman serum (rabbit) serum

ranging from 1:80 to 1:320 (by 100 per cent complement fixation and ring precipitation tests). A slight increase in titer was observed after a second stimulation (maximum titer 1:640). Two animals were used for each group of experiments and results of the experiments carried out with both sera in each group are presented.

*Kindly supplied by Dr. W. C. Liu, Department of Bacteriology, Harvard University.

a. Presence of serum proteins in the antigens: The antisera contained 2 main groups of antibodies: (α) against constituents of the vascular wall, and (β) against serum proteins. The absorption with human serum caused considerable loss of titer, but it allowed the distinction of the two groups of antibodies and made it possible to obtain sera directed specifically against the human vascular endothelium (Fig. 1). The serum proteins present in the antigens could not be characterized. When the antibodies were tested against human serum on a double agar diffusion plate, several precipitation bands were observed (Fig. 2). Multiple serum bands make Fig. 2 rather confused, yet the band due to endothelial antigens is still evident. The presence of human serum in the antigens was confirmed by the reaction of rabbit antihuman serum antibodies with the antigens under study (Table II).

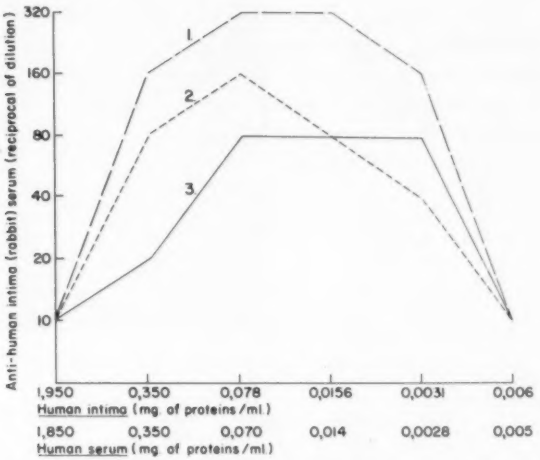


Fig. 1.—Titration of antihuman intima (rabbit) serum. Complement fixation test (100 per cent hemolysis): (1) native immune serum against human intima antigen; (2) immune serum absorbed with human serum against human intima antigen; (3)native immune serum against human serum. Antihuman intima rabbit serum contains two groups of antibodies directed (a) against intima and (b) against human serum.

TABLE II. PRESENCE OF HUMAN SERUM PROTEINS IN EXTRACTS FROM HUMAN VESSELS. COMPLEMENT FIXATION TEST (100 PER CENT HEMOLYTIC UNITS)

ANTIGEN	HUMAN SERUM	TOTAL INTIMA EXTRACT	WATER-SOLUBLE EXTRACTS			
			INTIMA	MEDIA	ADVENTITIA	PLACENTAL VESSELS
Antihuman serum (rabbit)	640	160	320	320	160	320

Serum proteins were present in all vessel extracts. Figures are the reciprocal of the maximum dilution of serum giving no hemolysis with the optimal dilution of antigen. This applies to all experiments when 100 per cent hemolysis complement fixation test was used.

b. Specific localization of the antibodies: The fluorescein tagging technique described demonstrated the localization of the antiendothelium antibodies at the

level of the endothelial layer of the intima and as bright scattered droplets in the adventitia of vessels (Fig. 3). Fluorescence in the kidney was localized not only in the vessels but also in the parietal sheath and areas of the visceral sheath of Bowman's capsule (Fig. 3). Endothelium and adventitia of the vessels were stained in the liver (as in other organs), but not the bile ducts. Antisera against water-soluble fraction and homogenate of the intima and antisera against adventitia and placental vessels showed the same localization. No significant difference was found using frozen rather than formalin-fixed sections. In the latter, however, the structure of the tissue was better preserved and more easily identified. No specific green fluorescence was noted in any of the control sections prepared, but only the blue-grayish spontaneous autofluorescence of the tissues. Thus, such sections are not shown.

c. Properties of the specific endothelial antigens: The antiserum against total extract of human endothelium still contained antibodies directed against the original antigens after absorption with human serum. The antiserum showed similar titer against extracts prepared from the adventitia and small placental vessels. Since it was impossible to isolate capillary tissue, small placental vessels were considered a closer representative of the capillary bed (Table III). No detectable reaction was observed against extracts from the media.

TABLE III. PRESENCE OF SPECIFIC VASCULAR ANTIGENS IN EXTRACTS FROM HUMAN VESSELS. COMPLEMENT FIXATION TEST (100 PER CENT HEMOLYTIC UNITS)

ANTIGEN	HUMAN SERUM	TOTAL INTIMA EXTRACT	WATER-SOLUBLE EXTRACTS			
			INTIMA	MEDIA	ADVENTITIA	PLACENTAL VESSELS
Antihuman endothelium native (rabbit) serum	160-320	640-640	320-320	160-320	320-320	320-160
Antihuman endothelium after absorption with human serum	0-0	160-80	160-80	0-0	160-80	160-80

Persistence of reaction even after absorption with human serum shows presence of specific vascular antigens in the total extract of intima and in water-soluble extracts of intima, adventitia, and placental vessels, but not of media.

The presence of antigenic substance in different organs or different fractions of the same organ can be demonstrated either by testing the cross reactivity of an antiserum with different organs or fractions, or by preparing different antisera for each organ or organ fraction and comparing the activity of sera thus obtained against each other. The properties of the antisera produced against the 3 layers of the aorta and the placental vessels are compared in Table IV. It would appear that most antisera contained antibodies directed against the endothelial antigen and against human serum proteins as well. The antiserum against the media extract, however, contained only antibodies against serum proteins and, after absorption, it became inert. These results could be explained best by postulating that endothelial antigens were present in the total vessel extract, as well as in the water-soluble extracts from the intima and in the water-soluble extracts of the adventitia and small placental vessels.

d. Relationship to Forssman antigens and blood group substances: The antisera were tested for Forssman antibodies,[11] in view of the relationship between Forssman and Forssman-like substances and blood group factors. Titers were in the normal range, thus excluding a relationship between human vascular antigens and Forssman antigens. The sera were matched against a series of human red cells of the major groups. Titers were in the range of normal (1:8 to 1:16) with no difference in any of the blood groups. This finding would seem to exclude the presence of group-specific agglutinins and of immune-type heteroagglutinins.

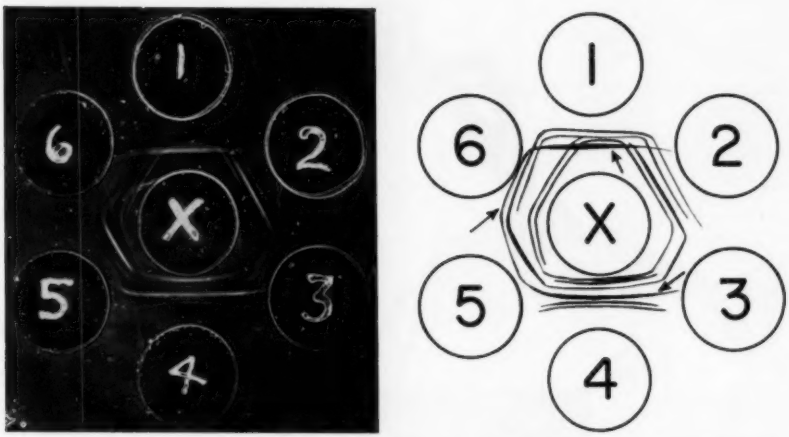


Fig. 2.—Agar precipitation plate. The central well is filled with antihuman endothelium (rabbit) serum. The peripheral wells contain the following antigens: (1) intima (water-soluble extract, 1.7 mg. protein per milliliter), (2) diluted human serum (1.34 mg. protein per milliliter), (3) liver (water-soluble extract, 1.1 mg. protein per milliliter), (4) placenta vessels (water-soluble extract, 1.22 mg. protein per milliliter), (5) kidney (water-soluble extract, 1.1 mg. protein per milliliter), (6) spleen (water-soluble extract, 1.58 mg. protein per milliliter). All antigens contained several human serum proteins. The arrows in the line drawing on the right point to an identity line present only with vascular antigens, kidney, and spleen; it is absent with serum proteins and liver extract.

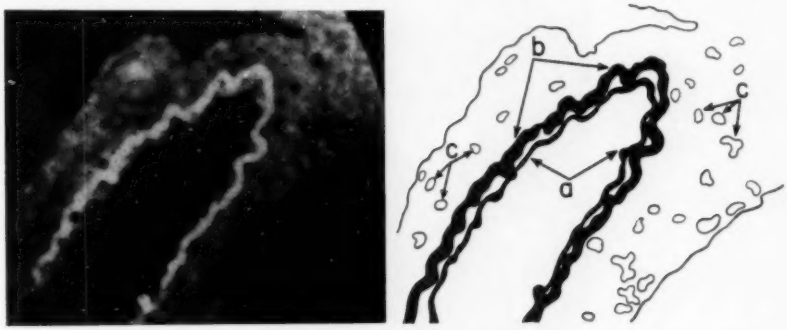


Fig. 3*A*.—Localization of anti-intima antibody by means of the fluorescent technique. Large vessel of human spleen. *a*, Endothelial lining, specific green fluorescence. *b*, Media, brilliant grayish autofluorescence. *c*, Adventitia, bright fluorescein droplets within the connective tissue. (Magnification of photomicrograph ×300; reduced 2/7.)

Fig. 3B.—Localization of anti-intima antibody by means of fluorescent technique. For legend see Fig. 3A. (Magnification of photomicrograph ×1,000; reduced ⅛.)

TABLE IV. ACTIVITY OF RABBIT SERA PREPARED AGAINST DIFFERENT CONSTITUENTS OF THE VASCULAR WALL. COMPLEMENT FIXATION (50 PER CENT HEMOLYTIC UNITS)

	ANTIGEN (UNITS PER MILLILITER)					
			WATER-SOLUBLE EXTRACTS			
ANTISERA	HUMAN SERUM	INTIMA HOMOG-ENATE	INTIMA	MEDIA	ADVENTITIA	PLACENTAL VESSELS
Anti-HE, native	1,080-1,180	1,200-1,300	1,380-1,500	1,080- 900	1,360-1,300	1,280-1,480
Anti-HE, absorbed	0-0	480-400	400-520	0-0	320-360	420-420
Anti-HI, native	1,180-1,280	1,300-1,200	1,500-1,520	900-1,080	1,300-1,240	1,480-1,540
Anti-HI, absorbed	0-0	400-480	520-520	0-0	360-320	420-480
Anti-HM, native	1,020- 980	960-1,040	1,040- 960	1,080-1,020	1,000-1,120	960-102
Anti-HM, absorbed	0-0	0-0	0-0	0-0	0-0	0-0
Anti-HA, native	1,160-1,280	1,100-1,420	1,120-1,280	1,000- 980	1,240-1,320	1,160-980
Anti-HA, absorbed	0-0	240-360	320-420	0-0	360-420	240-320
Anti-HPV, native	1,200-1,120	1,340-1,200	1,130- 980	1,180-1,040	1,420-1,240	1,380-1,420
Anti-HPV, absorbed	0-0	320-280	280-300	0-0	200-240	280-360

After absorption with human serum each antiserum reacted against water-soluble extracts of intima and adventitia, but not of media. The antimedia serum was entirely negative.

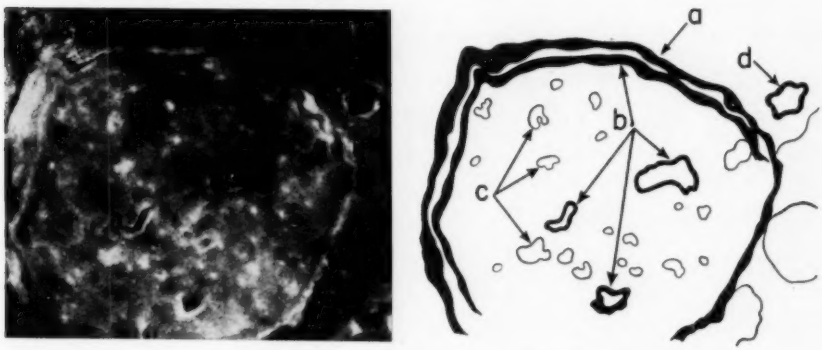


Fig. 3C.—Localization of anti-intima antibody by means of fluorescent technique. Kidney glomerules. *a*, Bowman's capsule, parietal. *b*, Bowman's capsule, visceral. *c*, Glomerular vessels, specific fluorescence. *d*, Paraglomerular vessel, bright fluorescence of the intima. Note also (arrows on line drawing) the hazy antifluorescence of the tubuli. (Magnification of photomicrograph ×1,000; reduced ⅕.)

TABLE V. CROSS REACTION OF ANTI-HI SERUM WITH HUMAN ORGANS. COMPLEMENT FIXATION (50 PER CENT HEMOLYTIC UNITS)

	ANTIGEN (UNITS PER MILLILITER)				
		WATER-SOLUBLE EXTRACTS			
ANTIBODY	HUMAN SERUM	INTIMA	KIDNEY	SPLEEN	LIVER
Anti-HI (rabbit) serum	1,280-1,280	1,520-1,520	1,320-1,400	1,380-1,280	1,020-1,120
Anti-HI (rabbit) serum, absorbed with human serum	0-0	520-520	280-380	320-420	0-0

Absorbed antihuman intima rabbit serum reacted with water-soluble extracts of intima, spleen, and kidney, but not liver.

e. Distribution of the vascular antigens in various organs: The antiendothelium sera were tested against human kidney, spleen, and liver tissue. The

TABLE VI. COMPARISON OF DIFFERENT TECHNIQUES (PRECIPITATION, COMPLEMENT FIXATION 100 PER CENT UNITS, COMPLEMENT FIXATION 50 PER CENT UNITS). ANTIBODY: ANTIHUMAN (SOLUBLE EXTRACT) (RABBIT) SERUM

			PRECIPITATION (RING TEST)*		COMPLEMENT FIXATION (100 PER CENT HEMOLYSIS)†		COMPLEMENT FIXATION (50 PER CENT HEMOLYTIC) (UNITS/ML.)		ANTIGEN / ANTIBODY RATIO‡ (MG. PROTEIN /ML.)
			NATIVE§	ABSORBED	NATIVE	ABSORBED	NATIVE	ABSORBED	
Antigens	Human serum		160-160	0-0	80-160	0-0	1,180-1,280	0-0	
	Human intima homogenate		Cloudy antigens		320-320	160- 80	1,300-1,200	400-400	0.420
	Water-soluble extracts	Intima	640-320	160-160	320-320	160-160	1,500-1,520	520-520	0.087
		Media	320-320	0-0	160-160	0-0	900-1,080	0-0	
		Adventitia	640-320	160- 80	320-320	160- 80	1,300-1,240	360-320	0.164
		Placental vessels	320-160	80-80	320-320	160- 80	1,480-1,540	420-480	0.244
		Kidney	Cloudy antigens		160-160	80-80	1,320-1,400	280-380	0.116
		Spleen	Cloudy antigens		320-160	80-80	1,380-1,280	320-420	0.746
		Liver	Cloudy antigens		160-160	0-20	1,020-1,120	0-0	

*Expressed as the reciprocal of the last dilution of serum giving a positive result.

†See Table II for expressions of results.

‡Optimal antigen concentration, with a standard 1:100 dilution of absorbed serum. (Results show good agreement between various techniques.)

§Native and absorbed antibody.

sera showed similar behavior in all respects. Thus, only the results of the experiments with one serum (anti-HI) will be described in detail. The unabsorbed serum reacted with all 3 organs. After absorption with human serum, cross reaction was present with kidney and spleen only, no longer detectable with liver. The titers were smaller than with the specific antigen and the optimal antigen/antibody ratio was much higher. The kidney extract reacted at a smaller concentration than the splenic extract, confirming that the reaction was roughly proportional to the vascularity of the organ (Table V). It seemed advisable to check the results with more than one serologic technique. Findings obtained with the 100 per cent hemolytic complement fixation and ring precipitation tests are compared in Table VI. Results were similar, although the antigen/antibody ratio was less sharp in the ring test than with complement fixation. This might have been due to the fact that, in the interfacial test, the antigen and antibody react with each other as soon as they meet in the range of the optimal ratio. The spectrophotometric determination of complement-fixing activity (50 per cent hemolytic complement fixation) allowed a finer, semiquantitative evaluation of the extent of the cross reaction.

TABLE VII. IMMUNOLOGIC RELATIONSHIP BETWEEN PLATELETS* AND VASCULAR ENDOTHELIUM. COMPLEMENT FIXATION (100 PER CENT HEMOLYSIS)†

	ANTIGEN			
ANTISERA	HUMAN SERUM	ENDOTHELIAL EXTRACT (TOTAL)	HUMAN PLATELETS	HUMAN PLATELETS (WASHED 12 TIMES)
Anti-HE (rabbit) serum	162-320	640-640	80-80	0-0
Anti-HE (rabbit) serum absorbed	0-0	160-80	0-0	0-0
Antihuman platelet (rabbit) serum	0-0	0-0	320-320	320-320

No apparent immunologic relationship was found between platelets and vascular endothelium.

*Results were identical using platelets from donors of blood group O or A.

†For expression of results, see Table II.

f. Antigenic relationship between platelets and endothelium: The presence of common antigens in platelets and endothelium has been postulated often,[16] but with insufficient evidence. It seemed, therefore, particularly interesting to elucidate this point by observing the existence of cross reaction between endothelium, platelets, and the respective antisera (Table VII). A slight cross reaction was evident between anti-HI serum and platelets. This disappeared after absorbing the antiserum with serum proteins or when platelets were used which had been washed in saline more than 10 times. Therefore, no common antigens could be demonstrated between platelets and endothelium.

g. Organ specificity: There was no cross reaction with endothelial antigens from dog, guinea pig, rabbit, calf, and sheep. Thus, no organ specificity could be demonstrated, although it could not be excluded on the basis of these experiments.

h. Effects of the intradermal injection of the immune serum in man: Antihuman intima rabbit serum was filtered through Seitz filters without loss of titer. Two-tenths milliliter of serum diluted 1:10 with saline was then injected intradermally in a man with terminal reticulum cell sarcoma, without response. Forty hours later, 0.2 ml. of undiluted serum was again injected intradermally. Equal volumes of normal rabbit serum and of antihuman globulin rabbit serum were used as controls in adjacent areas. An area of erythema developed at the site of injection of the antivessel serum but skin punch biopsies from all areas 10 hours after the inoculation were uniformly negative.

2. Precipitating Factors in the Serum of Patients With Anaphylactoid Purpura and Arteritis.—A factor precipitating in the presence of an extract of human vessel wall has been found in the serum of patients of anaphylactoid purpura and periarteritis nodosa.[4] Accordingly, sera from such patients were tested against the water-soluble extract of intima of human aorta, the antigenic properties of which have now been demonstrated, and several positive results were obtained (Table VIII). In order, however, to establish the specificity of

TABLE VIII. PLATE PRECIPITATION TEST AGAINST HUMAN GAMMA GLOBULIN AND AGAINST WATER-SOLUBLE ENDOTHELIAL EXTRACT IN PATIENTS WITH SOME HEMATOLOGIC DISORDERS

PATIENT	SEX	AGE	DIAGNOSIS	ENDOTHELIAL EXTRACT	GAMMA GLOBULIN
E. F.	M.	61	Periarteritis nodosa	+	+
M. C.	F.	48	Periarteritis nodosa	+	+
J. M.	M.	11	Anaphylactoid purpura	+	+
G. C.	M.	35	Anaphylactoid purpura (lupus)	+	+
G. R.	M.	17	Anaphylactoid purpura	+	+
E. L.	F.	69	Anaphylactoid purpura	+	+
E. R.	F.	60	Anaphylactoid purpura	–	–
L. P.	F.	40	Scleroderma	+	+
M. Z.	F.	38	Scleroderma	+	+
J. B.	M.	48	Dermatomyositis	+	+
E. O'C.	F.	22	Lupus erythematosus	+	+
S. O'N.	F.	72	Lupus erythematosus	+	+
E. M.	M.	77	Lupus erythematosus	+	+
R. T.	F.	40	Idiopathic thrombocytopenic purpura (lupus)	+	+
M. T.	F.	11	Idiopathic thrombocytopenic purpura	–	–
A. S.	M.	17	Acute lymphocytic leukemia	–	–
S. M.	M.	50	Chronic myelogenous leukemia	–	–
F. E.	M.	26	Lymphosarcoma	–	–
E. O.	M.	65	Acute myelogenous leukemia	–	–
M. G.	M.	20	Hypochromic anemia	–	–

the reaction, the agar precipitation technique was used (Fig. 4). One well was filled with the endothelial antigen, and the neighboring wells were filled with gamma globulin* and with soluble antigen from placental vessels. The central well was filled with serum, containing 1,000 units of penicillin per milliliter. Fig. 4 shows a typical "identity reaction" between the endothelial antigen and the gamma globulin. Thus, the precipitating factor present in the sera of some patients with vasculitis was reactive, not with the specific endothelial antigen, but rather with the serum proteins present in the extracts. In this respect,

*Kindly supplied by the American Red Cross.

it showed a behavior similar to the "rheumatoid arthritis factor" which has been found in the sera of patients with this disease and other disorders of the collagen.[17] Therefore, experiments were extended to the sera of patients with some disease of collagenous tissue. Most of them showed on the agar plate the same "identity reaction" which is found in the sera of patients with anaphylactoid purpura and periarteritis nodosa. A summary of these findings is presented in Table VIII.

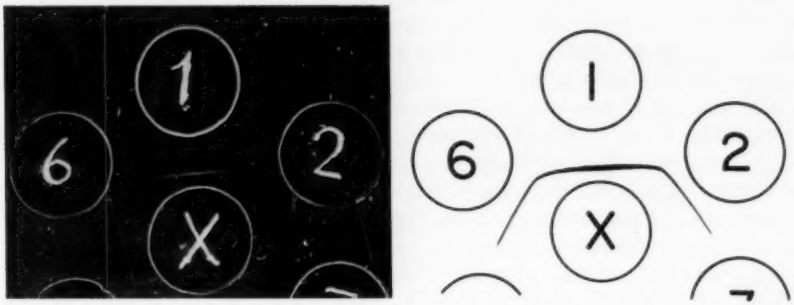


Fig. 4.—Precipitation reaction of the serum from a patient with anaphylactoid purpura (well X) against (1) endothelial antigen (water-soluble extract, 1.48 mg. protein per milliliter), (2) γ-globulin (1.32 mg. protein per milliliter), and (6) placental vessels (water-soluble extract, 2.44 mg. protein per milliliter). This figure shows identity reaction with the 3 antigens. Similar results were obtained with different degree of intensity with the sera showing positive reaction in Table VIII. Reactions against saline, serum albumin, and sodium penicillin (1,000/ml. units) were negative and are not shown in the figure.

DISCUSSION

The presence of antigenically active substances in the blood vessels of the rat was shown by Pressman and associates.[18] These authors used a fraction from rat liver that did not pass through a Delaphine press. Besides endothelium, however, their antigen contained many constituents of the connective tissue as well as bile ducts. Our experiments more clearly show the antigenic property of human vessels; they also indicate the particular distribution of these antigens, which was confirmed by experiments with fluorescein-tagged antibody techniques. Endothelial antigens were present in the intima and in the adventitia of the aorta, and in the endothelial lining of the small vessels isolated from the placenta. They were absent in the elastic structure of the vessels, a finding which confirms the lack of specific antigens in the elastic and smooth muscular tissues.[19] Finally, they were found in the kidney and spleen, but not in the liver tissue, a distribution roughly proportional to the vascular content of the organs. The absence of reaction between antiendothelium serum and liver extract did not signify the absence of endothelial antigens in this organ, and may only indicate their relatively lower concentration in the liver, as compared to kidney and spleen. It should be remembered that in the complement fixation test it is not possible to increase the concentration of the antigens above a certain limit (about 5 mg. per milliliter in the case of liver extract) because of the anticomplementary activity of the organ extract. It is of interest that Tanaha and Ledue[20] have recently confirmed earlier observations[21] that, in Forssman-positive species,

the Forssman antigen is localized in the intima and adventitia of blood vessels. They have postulated that, had this antigen a biologic significance, a corresponding substance may be detected in Forssman-negative species. On the basis of its localization the endothelial vascular antigen may represent in man the homologous of the Forssman antigen.

While human endothelium was antigenic, it presented no characteristics of organ specificity but, rather, of strong species specificities. The latter finding does not support the hypothesis of the autoimmune nature of vasculitis. Other observations on which are based the theory of vasculitis as an autoimmune disorder are made doubtful by our experiments as well. Thus, as mentioned, a precipitation reaction has been observed when the serum of patients with anaphylactoid purpura and periarteritis nodosa comes in contact with an extract of human vessel wall.[4] When this reaction is analyzed with more specific techniques, it becomes apparent that the vascular antigens used are contaminated significantly with serum proteins. This is practically unavoidable because of the source of the material and of the interval between death and collection of organs, which may allow imbibing of dead tissues by plasma proteins. Moreover, the connective tissue contains a considerable amount of serum proteins, representing an extravascular protein pool.[22] It may then be stated that the precipitating factors against endothelium extracts detected in the serum of patients with anaphylactoid purpura and periarteritis nodosa may not be specific and represent agents directed instead against components of serum. Studies with the double diffusion agar precipitation plate technique suggest that the factor in the sera of patients with periarteritis nodosa and anaphylactoid purpura may be in fact, identical to the rheumatoid arthritis factor. This agent has been demonstrated recently in the serum of patients with rheumatoid arthritis and collagen diseases and precipitates gamma globulin.[17]

Other supporting evidence for the autoimmune theory of the vasculitis syndrome has been the reproduction of a similar syndrome in animals by means of the injection of antiendothelium serum, as described by Katsura[2] and by Clark and Jacobs.[3] Moreover, Israel and co-workers[6] have described the reproduction of anaphylactoid purpura in guinea pig by the subcutaneous injection of the antiguinea pig vessel. It is doubtful whether these experiments using heterologous sera have any bearing on the discussion of a potential autoimmune mechanism. Moreover, we have repeated most of these experiments in this laboratory,[7] and we have either failed to confirm them or have been able to offer an alternative explanation. Bernard's results may be explained on the basis of an anaphylactoid reaction produced by the injection in guinea pigs of anti-Forssman antibodies. The injection of antiendothelium serum is followed by anaphylactoid-like shock in rabbits and guinea pigs and by delayed nephritis in dogs. In no instance may hemorrhagic lesions nor vasculitis be produced. In our studies, no vasculitis developed in the area of intradermal injection of antiendothelium serum in man. Unfortunately, this was a single experiment and the possibility of any larger experience is made unlikely by the difficulty of obtaining suitable volunteers.

Thus, the autoimmune theory of vasculitis is not substantiated by any of the experimental findings. This does not exclude its nature of immune disease. An analysis of the "immune" diseases involving the formed elements of the blood indicates that they may be produced by 3 major mechanisms[23]: (a) an autospecific antigen-antibody reaction, where the insulted cell also contains the offending antigen. In this case the resulting antibody is specifically directed against the damaged cell. This group includes the true autoimmune hematologic disorders, such as some instances of autoimmune thrombocytopenic purpura[24]; (b) a specific but not autospecific antigen-antibody reaction, where the cell involved, however, may not contain the offending antigen, and the formed elements of the blood are damaged in the course of a nonautospecific antigen-antibody reaction. The hemolytic anemia and thrombocytopenic purpura seen in the course of collagen disorders may be classified in this group; (c) hypersensitivity reactions, to which possibly belongs thrombohemolytic thrombocytopenic purpura. Anaphylactoid purpura and other forms of vasculitis may belong to the second group of this classification.

SUMMARY

1. Two major groups of antibodies were found in the antisera produced in rabbits against human vascular endothelium extracts. One, reacting with serum proteins, could be removed by absorption with human serum. By precipitation, complement fixation and other techniques, the remaining antivessel antibodies reacted against the specific endothelial antigens and extracts from kidney and spleen (highly vascularized organs) but not against liver. By the use of the fluorescein tagging technique, the antiendothelial antibody was localized primarily at the level of the endothelial layer of the vascular intima.

2. Antisera could be also prepared against the adventitia, but not against the media from human aorta. Vascular antigens from the entire aortic wall induced formation of specific antibodies against the adventitia and intima constituents only. The antigens proper to the vascular wall appeared unrelated to platelet antigens and showed no organ specificity. By virtue of their localization, the vascular antigens might represent the homologous substance of the Forssman antigens in species considered to be Forssman-negative (man).

3. Although some components of the human vascular wall are certainly heteroantigenic, the intradermal injection of the specific rabbit antiserum in man was not followed by significant reaction, either visible or by skin biopsy findings.

4. An analysis by double diffusion agar precipitation plate of the previously described precipitin reaction between the serum from patients with periarteritis nodosa and anaphylactoid purpura and an extract from the vascular wall showed that this reaction is nonspecific and due to the presence in the serum of these patients of a factor capable of precipitating gamma globulin (which is present in all vascular antigens).

5. Mainly on the basis of clinical observations, periarteritis nodosa, anaphylactoid purpura, and other forms of vasculitis are immune in origin. Nevertheless, no evidence could be found that they represent autoimmune disorders.

We wish to thank Dr. Albert H. Coons, Department of Bacteriology, Harvard Medical School, who kindly reviewed some of the sections stained with fluorescein-tagged antibodies and offered helpful suggestions and criticism.

REFERENCES

1. Stefanini, M.: Immuno-hematologic Disorders: A Theory of Disease, Proc. Inst. Med. Chicago **20**: 335-346, 1955.
2. Katsura, H.: Experimental Study on Purpura of Guinea Pigs. Purpura by the Anti-haemangio-Endothelial Cell Serum (Translated title), J. Osaka M. A. **22**: 373-385, 1923.
3. Clark, W. C., and Jacobs, E.: Experimental Nonthrombocytopenic Purpura: A Review of the Japanese Literature, With Preliminary Confirmatory Report, Blood **5**: 320-328, 1950.
4a. Stefanini, M., and Mednicoff, I. B.: Demonstration of Antivessel Agents in Serum of Patients With Anaphylactoid Purpura and Periarteritis Nodosa, J. Clin. Invest. (Abst.) **33**: 967, 1954.
4b. Stefanini, M.: Purpura Immunologique: une nouvelle entité pathogénique? Congrès Français de Medicine, XXX^e Session, Alger, 1955, pp. 165-177.
5. Steffen, C.: Weitere Untersuchungen und Überblick über die verschiedenen Immunologish Bedingten Formen der Hämorragischen Diathese, Wien. Ztschr. inn. Med. **37**: 453-464, 1956.
6. Israel, L., Mathe, G., and Bernard, J.: Sur la syndrome de Schönlein-Henoch. I. Reproduction expérimentale par un immun-sérum: II. Test décelant le pouvoir capillaro-toxique de l'immun-sérum et du sérum de malades, Rev. franç. étud. clin. et biol. **1**: 57-66, 1956.
7. Piomelli, S., Mele, R. H., and Stefanini, M.: To be published.
8. Ueno, S., Mele, R. H., and Stefanini, M.: Studies on Platelets XXIII. Organ specificity of Platelets, Proc. Soc. Exper. Biol. & Med. (In press.)
9. Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M.: The Activating Effect of Magnesium and Other Cations on the Hemolytic Function of Complement, J. Exper. Med. **84**: 535-548, 1946.
10. Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M.: Quantitative Studies of Complement Fixation. I. A Method, J. Immunol. **59**: 195-206, 1948.
11. Kabat, E. A., and Mayer, M. M.: Experimental Immunochemistry, Springfield, Ill., 1948, Charles C Thomas, Publisher, p. 135.
12. Boyd, W. C.: Influence of Character of Antibody Upon Velocity of Flocculation, J. Exper. Med. **74**: 369-386, 1941.
13. Ouchterlony, O.: Antigen-antibody Reactions in Gel and the Application of This Phenomenon in the Laboratory Diagnosis of Diphtheria, Thesis, Stockholm, 1949. (Quoted by: Wilson, M. W., and Pringle, B. H.: Experimental Studies of the Agar Plate Precipitation Test of Ouchterlony, J. Immunol. **73**: 232-243, 1954.)
14. Markham, R.: A Steam Distillation Apparatus Suitable for Micro-Kjeldahl Analysis, Biochem. J. **36**: 790-791, 1942.
15. Watson, B. K., and Coons, A. H.: Quoted by Kaplan, M. H.: Immunologic Studies of Heart Tissue, J. Immunol. **80**: 254-267, 1958.
16. Dausset, J.: Immuno-hématologie biologique et clinique, Paris, 1956, Ernest Flammarion, p. 456.
17. Epstein, W. V., Engleman, E. P., and Ross, M.: Quantitative Studies of the Precipitation and Agglutination Reaction Between Sera of Patients With "Connective Tissue" Diseases and a Preparation (Cohn Fraction II) of Human Gamma Globulin, J. Immunol. **79**: 441-449, 1957.
18. Pressman, D., Sherman, B., and Korngold, L.: The Zone of Localization of Antibodies. XIII. The "In Vivo" Localization of Anti-Liver Blood Vessel in the Rat, J. Immunol. **67**: 493-500, 1951.
19. Fleisher, M. D., and Arnstein, N.: Specificity of Anti-Organ Sera, J. Immunol. **6**: 223-224, 1921.
20. Tanaha, N., and Leduc, E. H.: A Study of the Cellular Distribution of Forssman Antigen in Various Species, J. Immunol. **77**: 198-212, 1956.
21. Halber, W.: Untersuchungen über das Forssmansche Antigen in Zusammenhang mit der Theorie der entspchung der Antikörper, Ztschr. Immunitätsforsch. **39**: 282-292, 1924.
22. Gitlin, D., Landing, B. H., and Whipple, A.: The Localization of Homologous Plasma Proteins in the Tissue of Young Human Beings as Demonstrated With Fluorescent Antibodies, J. Exper. Med. **97**: 163-176, 1953.
23. Stefanini, M., Piomelli, S., and Mele, R. H.: Immune-Hematologic Disorders of Childhood, *in* Schick, B., Festschrift, edited by W. Kaufman, 1959.
24. Kistner, S. A., and Stefanini, M.: Studies on Platelets XVII. An Experimental Study of the Development of Platelet Antibodies, J. Lab. & Clin. Med. **48**: 846-865, 1956.

SERIAL DETERMINATIONS OF SERUM GLYCOPROTEINS AND PROTEINS IN THE EARLY STAGES OF EXPERIMENTAL TUBERCULOSIS

HISAKO NISHIHARA, D.M.Sc., SEYMOUR FROMAN, PH.D., AND HENRY E. WEIMER, PH.D.
LOS ANGELES, CALIF.

CHANGES in the concentrations of the protein-bound carbohydrates and proteins of serum in clinical and in experimental tuberculosis have been the subject of numerous investigations. The decrease in serum albumin, the increase in the serum globulins, the elevation of the seromucoid fraction and the total serum glycoproteins are well documented and have been summarized in recent reviews.[1-5] How early in the course of infection these changes occur, however, is not well established. Previous investigations have been primarily concerned with alterations in the serum glycoprotein and protein patterns in well-established infections and with the effects of therapy. The present study was initiated to investigate the rate and extent of the serum changes in the early stages of an experimental infection in guinea pigs.

MATERIALS AND METHODS

Sixty-eight normal virgin guinea pigs were found to be nonreactors to the intracutaneous injection of 0.1 ml. of a 5.0 per cent solution of old tuberculin. A blood sample was then obtained by cardiac puncture under light ether anesthesia. One milliliter of blood per 100 Gm. of body weight was removed. Four weeks later the animals were inoculated subcutaneously in the inguinal region with 0.1 ml. of a suspension containing 0.1 mg. (moist weight) of *Mycobacterium tuberculosis*, strain B88.* Additional guinea pigs to a total of 88 were tested with tuberculin and inoculated. The animals were housed in an air-conditioned room in groups of 5 to 8 and were maintained on a diet of Purina rabbit pellets, supplemented with fresh greens, and tap water. The animals were again tested with tuberculin 2 days prior to autopsy. Groups of the guinea pigs were sacrificed 8, 15, 19, 29, 41, and 50 days following infection by exsanguination from the heart while under ether anesthesia. Skin tests were read and the extent of macroscopic tuberculosis determined by procedures previously reported.[6] Hematologic and chemical determinations were made by methods described in earlier communications.[7, 8] The mean, standard error of the mean, *t*, and probability values were calculated by standard statistical methods.[9]

From the Department of Infectious Diseases, School of Medicine, University of California, Los Angeles and Olive View Sanatorium, San Fernando, Calif.

Supported in part by grants from the Tuberculosis and Health Association of Los Angeles County.

A preliminary report was presented at the 43rd annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April, 1959.

Received for publication Jan. 21, 1959.

*This strain was originally isolated from a patient with active pulmonary tuberculosis by Dr. C. Richard Smith, Barlow Sanatorium, Los Angeles.

RESULTS

Due to the variability in the final weights of the infected groups, the data for the normal guinea pigs were originally subdivided into 3 groups according to animal weight. The mean weights for the groups were 846, 742, and 586 grams, and they were comprised of 21, 24, and 23 animals, respectively. No significant chemical or hematologic differences were observed among the groups. The data for the normal subgroups, therefore, were pooled. It may be concluded that the observed differences in the inoculated groups represent the systemic effects of infection. Inasmuch as no significant differences occurred between the groups autopsied 41 and 50 days after infection, the results for these groups were also pooled.

The effects of progressive infection on the average weight, hemoglobin, and hematocrit values are summarized in Table I. Transient fluctuations occurred in plasma volume as indicated by hematocrit values. It may be inferred, however, that the observed increases in serum components were not due to hemoconcentration. A consistent decline in hemoglobin values was noted in the groups with more extensive disease.

TABLE I. GENERAL AND HEMATOLOGIC DATA

GROUP (DAYS POST-INFECTION)	NO. OF ANIMALS	INITIAL WEIGHT* (GM.)	FINAL WEIGHT* (GM.)	HEMOGLOBIN* (GM. %)	HEMATOCRIT* (%)
0 (normal)	68		722 ± 13.9	12.9 ± 0.10	41 ± 0.3
8 days	22	772 ± 32.9	775 ± 30.2	12.7 ± 0.14	40 ± 0.4
15 days	16	749 ± 27.9	713 ± 32.0	13.0 ± 0.21	43 ± 0.6†
19 days	18	737 ± 37.4	737 ± 34.4	12.0 ± 0.18†	39 ± 0.5†
29 days	10	830 ± 15.6	851 ± 22.9	11.8 ± 0.13†	39 ± 0.5†
41 and 50 days	22	788 ± 27.5	733 ± 27.5	11.5 ± 0.18†	41 ± 0.5

*Including standard error of the mean.
†P = <0.01.

The results of tuberculin tests and autopsy findings are presented in Table II. The size of the inoculum employed was adequate to ensure a rapid conversion of dermal sensitivity and progressive disease. Early gross macroscopic changes were limited essentially to the spleen and lymph nodes.

The effects of tuberculous infection on the protein-bound carbohydrates of serum are shown in Table III. A significant increase in the concentration of total glycoprotein due to the elevation of globulin polysaccharide had occurred 8 days postinoculation. Over 50 per cent of the increase was attributable to the globulin subfraction, seromucoid. Maximum levels were attained 15 days following infection and remained relatively stable for the duration of the experiment, notwithstanding a progressive increase in the extent of tuberculosis. In the 8- to 15-day period, the increase in the extent of tuberculosis was paralleled by the elevation in total serum glycoprotein and the bound carbohydrates of the seromucoid and globulin fractions. Albumin polysaccharide values were little affected by the disease process, even though the protein component of the fraction decreased significantly (Table IV).

Protein changes (Table IV) in the subfractions of serum were more pronounced in the initial stage (0 to 8 days) of infection than those observed for the polysaccharide components. A marked hyperproteinemia not caused by hemoconcentration was found in the 50-day group. The A/G ratio had declined to a minimum value by the fifteenth day postinfection. No further changes in the distribution of the serum proteins occurred.

TABLE II. INDEX OF TUBERCULOSIS IN ACUTE EXPERIMENTAL INFECTION

GROUP (DAYS POST-INFECTION)	AVERAGE INDEX OF TUBERCULOSIS*					TUBERCULIN REACTION
	SPLEEN	LIVER	LUNGS	LYMPH NODES	TOTAL†	
8 days	0.5	0.0	0.0	0.0	0.5 ± 0.11	1.2
15 days	0.6	0.1	0.0	1.7	2.4 ± 0.30	1.1
19 days	1.0	1.0	0.1	1.5	3.6 ± 0.24	1.6
29 days	2.4	0.8	0.8	3.1	7.1 ± 0.41	4.0
41 and 50 days	2.0	1.5	1.8	2.6	7.9 ± 0.42	3.8

*Each organ (spleen, liver, lungs, and lymph nodes) was assigned a value of 0 to 4 according to extent of macroscopic tuberculosis. Maximum possible extent of disease in each animal was 16. Index of tuberculosis is the average total for each group.
†Including standard error of the mean.

TABLE III. SERUM PROTEIN-BOUND CARBOHYDRATE* CHANGES IN ACUTE EXPERIMENTAL TUBERCULOSIS

GROUP (DAYS POST-INFECTION)	TOTAL GLYCOPROTEIN† (MG. %)	SEROMUCOID POLY-SACCHARIDE† (MG. %)	ALBUMIN POLY-SACCHARIDE† (MG. %)	GLOBULIN POLY-SACCHARIDE† (MG. %)	A/G RATIO†
0 (normal)	116 ± 1.0	33 ± 0.4	25 ± 0.7	91 ± 1.1	0.27 ± 0.009
8 days	131 ± 2.9‡	41 ± 2.0‡	26 ± 1.1	105 ± 3.0‡	0.25 ± 0.014
15 days	165 ± 3.3‡	63 ± 2.7‡	27 ± 1.2	138 ± 2.9‡	0.20 ± 0.012‡
19 days	163 ± 4.1‡	62 ± 2.8‡	20 ± 1.1‡	143 ± 4.3‡	0.15 ± 0.010‡
29 days	154 ± 6.7‡	55 ± 4.2‡	23 ± 1.3	131 ± 6.6‡	0.18 ± 0.011‡
41 and 50 days	161 ± 4.0‡	61 ± 2.9‡	24 ± 1.0	137 ± 4.3‡	0.18 ± 0.010‡

*Hexose.
†Including standard error of the mean.
‡Statistically significant difference from normal values, P = <0.01.

TABLE IV. SERUM PROTEIN CHANGES IN ACUTE EXPERIMENTAL TUBERCULOSIS

GROUP (DAYS POST-INFECTION)	TOTAL* (GM. %)	SEROMUCOID* (GM. %)	ALBUMIN* (GM. %)	GLOBULIN* (GM. %)	A/G RATIO*
0 (normal)	4.7 ± 0.03	0.30 ± 0.003	3.0 ± 0.02	1.7 ± 0.02	1.76 ± 0.020
8 days	4.9 ± 0.07†	0.39 ± 0.019†	2.7 ± 0.07†	2.2 ± 0.04†	1.23 ± 0.043†
15 days	4.8 ± 0.07	0.55 ± 0.017†	2.4 ± 0.07†	2.4 ± 0.05†	1.00 ± 0.043†
19 days	4.7 ± 0.05	0.64 ± 0.033†	2.3 ± 0.09†	2.4 ± 0.05†	0.96 ± 0.062†
29 days	4.9 ± 0.15‡	0.55 ± 0.038†	2.5 ± 0.14†	2.4 ± 0.05†	1.04 ± 0.078†
41 and 50 days	5.3 ± 0.10†	0.59 ± 0.027†	2.6 ± 0.06†	2.7 ± 0.07†	0.96 ± 0.046†

*Including standard error of the mean.
†Statistically significant difference from normal values, P = <0.01.
‡P = <0.05 >0.01.

DISCUSSION

The current investigation has demonstrated that the serum glycoprotein and protein changes previously observed in well-established infections occur

quite early in the course of the disease. In attempting to interpret the data, several factors must be noted: the rate and extent of the serum changes, the relative stability of the serum glycoprotein, and protein patterns following the initial alterations notwithstanding a progressive increase in the average index of tuberculosis.

Several hypotheses reviewed by Winzler[1] have been advanced to account for increased concentrations of the serum glycoproteins in a number of physiologic and pathologic conditions: tissue injury, tissue degradation, tissue proliferation and repair, increased serum protein synthesis, and nonspecific stress. All of the above factors are present in acute experimental tuberculosis. It is suggested that the results obtained in the present study may represent primarily systemic manifestations of the inflammatory response.[10, 11] The many facets of this basic response to tissue injury and the lack of precise knowledge of the biochemical mechanisms involved still leave the cause of the alterations in the serum glycoprotein and protein patterns undefined.

SUMMARY

The systemic effects of acute experimental tuberculosis as reflected by alterations in the concentration and distribution of the serum glycoprotein and proteins have been investigated in adult female guinea pigs. The levels of the protein-bound carbohydrates and proteins in serum and in the seromucoid, albumin, and globulin fractions were determined in groups of animals sacrificed 8, 15, 19, 29, and 50 days following inoculation with 0.1 mg. *Mycobacterium tuberculosis*, strain B88. The results demonstrate that the acute infection is characterized by the following changes: (1) significant and rapid increases in the components of the seromucoid and globulin fractions; (2) rapid decline in the protein components of the albumin fraction; (3) no change in albumin polysaccharide values; (4) more pronounced changes in the protein components of the serum subfractions in the initial stage of the infection; and (5) stability of the serum glycoprotein pattern after 15 days' infection notwithstanding progressive spread of disease.

REFERENCES

1. Winzler, R. J.: Determination of Serum Glycoproteins, Methods of Biochemical Analysis, New York, 1955, Interscience Publishers, Inc., vol. 2, pp. 279-311.
2. Stary, Z.: Mucoproteins in Clinical Chemistry, Clin. Chem. **3**: 557-576, 1957.
3. Südhof, H., and Kellner, H.: Physiologie und Klinische Bedeutung Kohlenhydrathaltiger Korperstoffe, Bibliot. Paediat. **65**: 1-104, 1957.
4. Biserte, G.: Les glycoproteides du serum sanguin et de l'urine. Exploration et signification physiopathologique, Bull. Soc. chim. biol. **39** (Suppl. 3): 93-158, 1957.
5. Gutman, A. B.: The Plasma Proteins in Disease, Advances Protein Chem. **4**: 156-250, 1948.
6. Weimer, H. E., Boak, R. A., Bogen, E., Drusch, H. E., Miller, J. N., Moshin, J. R., and Carpenter, C. M.: Comparative Effects of Corticotropin and Cortisone on Experimental Tuberculosis, Am. Rev. Tuberc. **68**: 31-41, 1953.
7. Weimer, H. E., Quinn, F. A., and Nishihara, H.: The Effects of Repeated Bleedings on Serum Glycoprotein Concentrations in the Guinea Pig, Am. J. Physiol. **190**: 529-532, 1957.
8. Weimer, H. E., and Quinn, F. A.: Seromucoid (Mucoprotein) Determinations in Experimental Animals, Clin. chim. acta **3**: 419-422, 1958.
9. Snedecor, G. W.: Statistical Methods, ed. 5, Ames, 1956, Iowa State College Press.

10. Stidworthy, G., Payne, R. W., Shetlar, C. L., and Shetlar, J. R.: Objective Evaluation of Patients With Rheumatic Diseases. II. Paper Electrophoretic Studies of Serum Glycoprotein and Protein From Patients With Rheumatoid Arthritis, J. Clin. Invest. 36: 309-313, 1957.
11. Heppleston, A. G., and Keyser, J. W.: The Relationship of Tissue Formation and Destruction to the Serum Protein-Bound Carbohydrate Levels in Experimental Inflammation, Brit. J. Exper. Path. 38: 111-119, 1957.

THE RELATIONSHIP OF THE CATION AND WATER CONTENT OF RED BLOOD CELLS TO PLASMA COMPOSITION

Evidence of Cell Potassium Deficiency in Adrenocortical Insufficiency, Nephritis, and Hepatic Cirrhosis

H. G. Keitel, M.D.,* and H. B. S. Jones, B.S.
Bethesda, Md.

IT IS generally accepted that osmotic equilibrium is present between red cells and plasma.[1] Darrow and Yannet[2] reported that the monovalent cation concentration† (sodium plus potassium) varied directly and equally in plasma and red cells when the plasma concentration of electrolytes was suddenly altered. These data have been interpreted as indicating that osmotic equilibrium between red cells and plasma is predominantly governed by shifts in water secondary to the change in the concentration of sodium plus potassium. However, Christensen and Riggs[3] have shown that organic cations can enter red cells and replace potassium as the predominant cell cation, and Elkinton and associates[4] have presented evidence that the osmotic activity of cell base may change during certain metabolic diseases.

Van Slyke, Wu, and McLean[5] have shown that the concentration of chloride and of bicarbonate in red cells can be predicted when the plasma concentration of chloride, bicarbonate, and hydrogen ion are known. From their studies it would be predicted that the sodium plus potassium concentration of red cells may not change equally during acidosis and alkalosis. Furthermore, a requirement in their mathematical formation is a constancy of the red cell sodium and potassium contents.‡[5] Since profound alterations in the sodium and potassium concentration of red cells have been observed in human beings with electrolyte disturbances, including those with alterations of blood pH (7 to 12) and those in which the urinary excretion of organic cations have been demonstrated,[6] it was decided to re-examine the relationship of the red cell sodium plus potassium and sodium plus potassium plus chloride concentrations in a wide variety of diseases including those associated with electrolyte disturbances. From these observations, one might evaluate the role of sodium, potassium, and chloride in the maintenance of osmotic equilibrium between red cells and plasma.

From the Children's Medical Service, Massachusetts General Hospital, the Department of Pediatrics, Harvard Medical School, and the National Institutes of Health, Bethesda, Md.

This work was supported in part by a grant from the Commonwealth Fund of New York.

Presented as an abstract for the American Federation for Clinical Research, February, 1957.

Received for publication Dec. 24, 1958.

*Present address: Pediatric Department, Jefferson Medical College, 1025 Walnut Street, Philadelphia, Pa.

†Concentration = quantity per kilogram of cell water.

‡Content = quantity per kilogram of cell solids.

The data are also examined from another point of view. When cell *solids* are used as a basis of reference for water and electrolyte composition, one can quantitatively appraise the change in the *content* of these substances in cells.[13] The use of a liter or kilogram of wet red cells or of red cell water as a parameter tends to obscure the true change in the content of water and electrolytes of cells. When the electrolyte data were referred to cell solids, it was found that the red cell content of potassium was reduced in patients with Addison's disease and some patients with uremia; it had been thought that the potassium *content* of cells was usually increased in these diseases.[13-16]

MATERIALS AND METHODS

Thirty patients, presented in detail in Table I, had renal, cardiac, or hepatic disease; with the exception of one (Subject 21) they were edematous and on a diet containing from 10 to 50 mM. of sodium per day. Cases 1 to 7 had typical findings of uncomplicated nephrotic syndrome without nitrogen retention.[17] Cases 8 to 16 and 18 to 21 had other types of nephritis with nitrogen retention. Cases 22 to 26 had conjestive heart failure, and 4 cases (27 to 30) had advanced hepatic cirrhosis. With the exception of Cases 17 and 25, Subjects 10 to 30 were critically ill.

Four cases, presented in detail in Table II, were admitted with acute adrenal insufficiency. Two of them (31 and 32) had Addison's disease with a history of salt craving and pigmentation of many months' duration. Laboratory findings including markedly reduced urinary excretion of 11-oxy- and 17-ketosteroids and absence of blood corticosteroids (F) following the administration of adrenocorticotrophic hormone. The other two cases (33 and 34) had salt wasting associated with the adrenogenital syndrome. Treatment following admission for adrenal insufficiency consisted of DOCA, salt, cortisone, and diet ad libitum.

Two additional patients, one with the adrenogenital syndrome and the other with Addison's disease, were under treatment with cortisone and desoxycorticosterone acetate and were asymptomatic.

Two patients had congenital pseudodiabetes insipidus (Pitressin-resistant diabetes insipidus). One of them weighed 17.3 Kg. after acute water deprivation and shortly after drinking water to capacity weighed 20.5 Kg. (see Table III).

Eighteen patients were thought to have electrolyte disturbances; 4 of these had potassium deficiency as manifested by low serum potassium concentration and a large retention of potassium following potassium administration.* Nine had diabetic acidosis and, with the exception of one who died, all were restudied during convalescence. Five with miscellaneous diseases were thought to have a possible electrolyte disturbance on the basis of the history of vomiting.

The remaining patients, with few exceptions, had a normal composition of red cells and plasma. They included 3 patients with uncomplicated essential hypertension, 6 with diabetes mellitus but without acidosis, and 6 with chronic rheumatoid arthritis. Eleven patients had cancer; some of these were malnourished but none were cachectic. Ten patients had acute surgical conditions, and 11 others had miscellaneous medical diseases.

Chemical Methods.—For the reasons indicated elsewhere,[19] the direct method for red cell analysis was used to ensure accuracy. Blood samples of 10 to 20 ml. were drawn without the use of a tourniquet from the antecubital vein into silicone-coated syringes which contained less than 0.05 ml. of isotonic heparin. The blood was kept in the stoppered syringe at room temperature with negligible loss of CO_2. When CO_2 or pH was to be determined a separate sample of blood was kept iced under mineral oil.

*The balance and red cell data of these patients are presented elsewhere.[18]

TABLE I

CASE	DIAGNOSIS		PLASMA GM./KG. H_2O	PLASMA mM./KG. H_2O Na	PLASMA mM./KG. H_2O K	PLASMA Cl	PLASMA MG. % NPN	PLASMA pH	RED CELL GM./KG. SOLIDS H_2O	RED CELL mM./KG. SOLIDS Na	RED CELL mM./KG. SOLIDS K	RED CELL mM./KG. SOLIDS Cl
1	Nephrotic syndrome	1+ edema	918	148	2.8	115	34	7.43	1,880	36	269	163
2	Nephrotic syndrome	2+ edema	929	142	4.1	109	–	–	2,031	39	275	145
3	Nephrotic syndrome	2+ edema	927	145	4.9	115	45	–	1,942	37	264	157
4	Nephrotic syndrome	2+ edema	923	148	4.9	111	–	–	2,025	38	280	161
5	Nephrotic syndrome	2+ edema	928	149	4.1	110	14	7.42	1,947	31	247	163
6	Nephrotic syndrome	3+ edema	919	146	4.3	117	17	7.39	2,000	34	295	176
7	Nephrotic syndrome	4+ edema	940	149	5.3	–	–	–	1,983	45	283	148
8	Acute nephritis	4+ edema	928	152	8.3	111	285	7.24	1,861	29	256	161
9	Acute nephritis	2+ edema	923	151	7.4	105	155	7.30	2,051	39	259	146
10	Chronic nephritis	4+ edema	942	121	6.5	71	205	7.35	1,997	18	242	–
11	Chronic nephritis	2+ edema	913	140	5.2	101	250	7.32	1,815	29	230[o]	–
12	Chronic nephritis	3+ edema	930	133	5.9	108	114	7.35	2,225	21	285	173
13	Chronic nephritis	3+ edema	929	136	2.4	99	85	7.43	1,959	30	218	–
14	Chronic nephritis	3+ edema	922	145	5.4	111	170	7.27	1,751	34	216	183
15	Chronic nephritis	3+ edema	934	133	5.3	106	150	7.32	2,000	22	248	150
16	Chronic nephritis	2+ edema	911	155	5.1	122	87	7.40	1,985	26	266	164
17	Chronic pyelonephritis	2+ edema	915	148	4.6	100	39	7.40	1,998	30	278	–
18	Chronic pyelonephritis	2+ edema	918	146	5.7	100	62	7.48	2,008	23	277	127
19	Nephrosclerosis											
	(A) Deficiency	1+ edema	906	144	3.1	78	48	7.56	1,879	43	236	71
	(B) Post KCl R		905	151	4.7	106	–	–	1,887	29	258	137

20	Intercapillary glom. sclerosis	3+ edema	924	145	7.3	118	77	–	1,979	31	217	214
21	Intercapillary glom. sclerosis	No edema	915	143	5.4	109	66	7.48	1,934	48	258	145
22	Cardiac failure due to rheumatic valvulitis	4+ edema	919	147	6.8	107	120	7.35	2,100	41	289	161
23	Cardiac failure due to rheumatic valvulitis	2+ edema	926	134	6.5	92	37	7.43	2,092	20	293	118
24	Cardiac failure due to rheumatic valvulitis	1+ edema	900	153	4.4	95	35	7.38	1,906	45	257	104
25	Cardiac failure due to hypertension	(A) 4+ edema	927	144	5.8	106	23	7.43	2,189	37	299	160
		(B) Post R no edema	922	152	5.0	106	–	7.40	2,128	43	313	183
26	Cardiac failure due to coronary artery disease	2+ edema	931	138	4.5	102	120	7.47	2,118	32	272	161
27	Laennec's cirrhosis	4+ edema	950	115	6.1	91	20	7.42	2,163	22	239	133
28	Laennec's cirrhosis	4+ edema	952	139	2.7	96	14	–	2,065	36	233	91
29	Laennec's cirrhosis	4+ edema	915	135	4.3	103	15	7.35	2,050	23	276	147
30	Laennec's cirrhosis	4+ edema	942	129	5.7	104	38	–	2,221	32	250	–

NORMAL RED CELL CONTENT OF SODIUM AND POTASSIUM[19]
(MEQ./KG. RED CELL SOLID)

	SODIUM	POTASSIUM
Men	43.2 ± 3.9	267.9 ± 8.8
Women	35.9 ± 3.7	276 ± 10.7

TABLE II. RED CELL AND PLASMA COMPOSITION IN PATIENTS WITH ADRENAL INSUFFICIENCY AND FOLLOWING RECOVERY

PATIENT	DIAGNOSIS	DAYS AFTER R FOR CRISIS	PLASMA				RED CELLS			
			GM./KG.	mM./KG. H_2O			GM./KG. SOLIDS	mM./KG. SOLIDS		
			H_2O	Na	K	Cl	H_2O	Na	K	Cl
31	Addison's disease	0	932	131	5.4	87	1,842	27	234	102
		23	931	152	3.2	–	1,999	32	271	–
32	Addison's disease	0	912	140	4.8	98	1,995	28	249	133
		100	908	153	4.2	116	1,915	35	264	147
33	Adrenogenital syndrome, salt-waster	0	909	146	5.0	103	1,897	32	232	148
		12	913	156	4.9	112	1,832	39	248	148
34	Adrenogenital syndrome, salt-waster	0	905	145	5.5	114	1,995	35	246	150
		7	919	152	5.0	115	1,842	31	267	177

TABLE III. PLASMA AND RED CELL WATER AND ELECTROLYTE COMPOSITION FOLLOWING AN ACUTE CHANGE IN TOTAL BODY WATER CONTENT (PATIENT E. D., PITRESSIN-RESISTANT DIABETES INSIPIDUS)*

BODY WEIGHT (KG.)	PLASMA					RED CELLS								WHOLE BLOOD HEMATOCRIT
	GM./KG.	mM./KG. H_2O				GM./KG.	GM./KG. SOLIDS	mM./KG.						
								SOLIDS			H_2O			
	H_2O	Na	K	Cl	pH	H_2O		Na	K	Cl	Na	K	Cl	
17.3	886	190	4.9	130	7.41	655	1,894	50	300	174	26	159	91	43.9
20.5	920	142	4.8	100	7.40	692	2,247	46	304	157	20	136	70	41.9

*A large fall in sodium and potassium concentration of red cells was observed, but the red cell content of these substances did not exceed experimental variability. A 17 mM. reduction in the chloride content of red cells was observed which can be attributed to the change in plasma chloride concentration and not to a change in the blood pH. In the absence of other changes, only a small drop in hematocrit is to be expected as a result of a large increase in total body water content, the drop representing the difference in the water content of red cells (710 Gm. per liter) and of plasma (930 Gm. per liter).

A well-mixed aliquot was transferred immediately to a rubber-stoppered Wintrobe tube for hematocrit determination. An aliquot of blood, calculated from the hematocrit or whole blood hemoglobin determination, was then transferred to a silicone-coated, rubber-capped Butler-Cushman type constricted centrifuge tube. The specimens were centrifuged at about 4,100 r.p.m. (mean radius of gyration of 15 cm.) for 45 minutes at 20° C. After centrifugation the supernatant plasma and buffy layer were completely removed. The entire red cell sample was pipetted into a tared glass-stoppered weighing bottle and weighed. The sample was then diluted with an accurately measured volume of about 4 ml. of distilled water per gram of red cells. Thoroughly mixed aliquots of this solution were used for various analyses. A Barclay flame photometer, with an internal lithium standard, was used for the sodium and potassium analysis.[20] Chloride was determined by a modification of the Volhard method,[21] silver nitrate and nitric acid being added separately as recommended by Wilson and Ball,[22] and the CO_2 content was determined by the method of Van Slyke and Neill.[23] The water content was determined by drying specimens for 24 hours at 105° C.

Calculations and Methods of Presenting Red Cell Data.—The analytic values of cell constituents as grams or millimoles per kilogram of cells* were converted to quantities per kilogram of cell solids by dividing the former values by the fraction of solids per

*When data are presented in quantities per liter they may be converted to quantities per kilogram by dividing by 1.1, the approximate specific gravity of red cells. Amounts per liter of plasma are converted to amounts per kilogram of plasma by dividing by 1.025, the approximate specific gravity of plasma.

kilogram of cells. No correction was made for trapped plasma. The conversion from quantities per kilogram of cell solids to quantities per kilogram of cells, or cell water, is as follows:

$$(1)\quad \frac{\text{quantity}}{\text{Kg. cell}} = \frac{\text{quantity}}{\text{Kg. solids}} \times (1\text{-}W)$$

$$(2)\quad \frac{\text{quantity}}{\text{Kg. water}} = \frac{\text{quantity}}{\text{Kg. solids}} \times \frac{(1\text{-}W)}{(W)}$$

where W = water, as fraction of the weight cells.

It is assumed that red cell solids (which consist chiefly of hemoglobin) remain constant during the life of the erythrocyte. Therefore, an acute change in water or electrolyte content of red cells exceeding methodologic variability will be considered to be a true change.

Red cells which are compacted by the method described (vide supra) contain 2 ± 0.5 per cent plasma by volume[19]; therefore, the reported red cell sodium values are about 25 per cent higher than the true value. As the amount of trapped plasma varies from 1.5 to 2.5 per cent of the compacted cells and as the plasma sodium *concentration* of the patients being reported varied from a low of 115 mM. to a high of 159 mM., a change in the red cell sodium *content* less than 5 mM. (about 15 per cent) may not exceed methodologic variability. However, the maximum error from red cell potassium content resulting from variability of trapped plasma is about 0.5 per cent, and for the red cell water content about 1.3 per cent.

The coefficients of variation of multiple analyses on individual samples are reported elsewhere.[19]

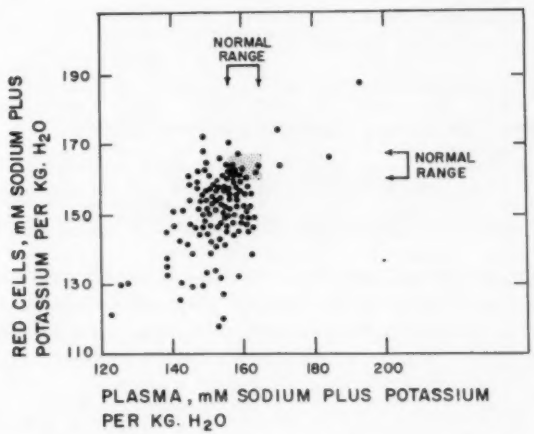


Fig. 1.—The sodium plus potassium (Na + K) concentration of red cells (Y) as related to the concentration of Na + K of plasma (X). The best-fitting line by least squares is: Y = 79.5 + 0.467 X. The 95 per cent confidence limits on the slope are 0.453 and 0.481. The shaded area includes the values found in 22 normal subjects.[19]

RESULTS

A. The Relationship of the Sodium, Potassium, and Chloride Concentration of Red Cells to Plasma Composition.—The sum of the (sodium plus potassium) concentration of red cells and of plasma of all patients studied is presented in Fig. 1. The predictability of the sodium plus potassium concentration of Y (red cells) has a fairly large standard deviation, for example, when X = 150,

Y is found to be 149.5 ± 17.8. These data cannot be interpreted as suggesting osmotic dysequilibrium or the presence of unknown organic solutes since labile anions (chiefly chloride) constitute a large fraction of the solute content of red cells and plasma.

Acidosis and alkalosis result in a redistribution of the freely diffusible electrolytes (chloride, bicarbonate, and hydrogen) between plasma and red cells according to Gibbs-Donnan equilibrium.[24] The increased solute *content* (primarily chloride ion) of red cells during acidosis results in an increase in their water content, with a decrease in the ratio of the cell to plasma sodium plus potassium concentration. An opposite change occurs during alkalosis.

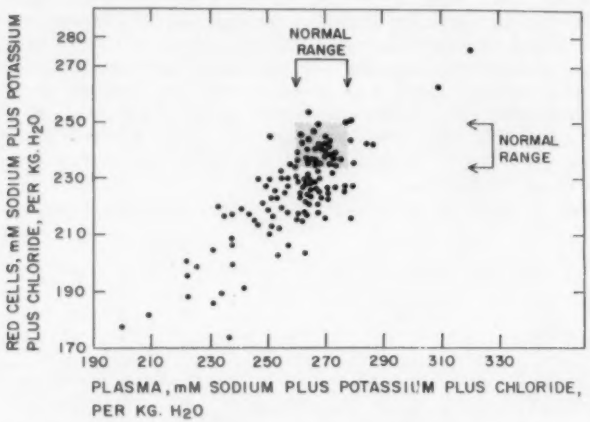


Fig. 2.—The sodium plus potassium plus chloride (Na + K + Cl) concentration of red cells (Y) as related to the concentration of Na + K + Cl of plasma (X). The best-fitting line by least squares is: Y = 17.7 + 0.803 X. The 95 per cent confidence limits for the slope are 0.687 and 0.919. The shaded area includes the values found in 22 normal subjects.[19]

It is not surprising, therefore, that the monovalent cation concentration of red cells varied independently of the plasma monovalent cation concentration in patients with metabolic disturbances, including those associated with alterations in blood pH. Fig. 2, which contains data of those patients presented in Fig. 1 in whom chloride measurements* were made, shows a direct, highly significant relationship between the sum of the sodium plus potassium plus chloride concentration of red cells and plasma (Y = 17.7 + 0.803 X). The predictability of the sodium plus potassium plus chloride concentration of Y (red cells) has a fairly low standard deviation; for example, when X = 250, Y is found to be 218.6 ± 4.2. The presence in red cells and plasma of differing concentrations of bicarbonate, phosphate, magnesium, and calcium, together with analytic errors, could easily account for the relatively small variability of almost all points.†

*Seventy per cent or more of the diffusible anions are usually comprised of chloride.

†Varying concentrations of glucose and urea do not usually result in an osmotic gradient between red cells and plasma in human beings since at most concentrations these solutes are found in equal concentrations in the water phases.[34]

Fig. 3 indicates that the blood pH may be the chief factor which governs the red cell to plasma (sodium plus potassium) concentration ratio. The points include all patients presented in Figs. 1 and 2 on whom pH measurements were made. It is seen that at a pH of 7.1 the cell to plasma concentration ratio of sodium plus potassium is about 0.85 whereas at a pH of 7.5, the ratio is about 1.05.

An increase in the plasma chloride concentration at any given pH should also be accompanied, according to the Gibbs-Donnan equilibrium, by a shift of chloride and therefore of water into red cells; a reduction of the red cell to plasma concentration ratio of sodium plus potassium will result, therefore.

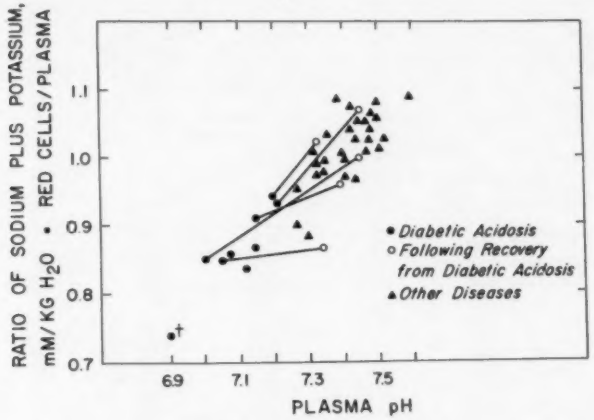


Fig. 3.—The relationship of the sodium plus potassium concentration ratio of red cells to plasma at varying pH.

The findings of the water-deprived patient with diabetes insipidus presented in Table III appear to illustrate such an occurrence since a 17 mM. increase in the red cell chloride content was observed during hyperchloremia secondary to water deprivation, and the sodium plus potassium concentration ratio of red cells to plasma fell in the absence of a change in the pH.

B. The Red Cell Sodium, Potassium, and Water Content: Relationship to Plasma Composition.—The apparent relationship of the red cell cation and water content to plasma ionic and water composition must be considered with the following in mind: the length of time that a plasma abnormality may have been present, the opposing or summating effect resulting from two independent changes, and the effect of unknown or unmeasured substances which vary directly with one of the measured substances. It is appropriate to inspect the apparent plasma and red cell ionic interrelations with these limiting factors in mind.

1. The relationship of the plasma sodium concentration to the red cell monovalent cation content: The effect of acute hypo- and hypernatremia on red cell composition is best illustrated by the patient with pseudodiabetes insipidus

(Pitressin-resistant diabetes insipidus) whose body weight increased 19 per cent following maximum ingestion of water (see Table III). The blood pH and hematocrit remained virtually unchanged.*

The measured change in red cell water content was close to the calculated change assuming that the retained water was distributed (proportionately) to all body fluid phases and that 60 per cent of body weight was water. A large change in the *concentration* of sodium and potassium of red cells was noted but the change in the sodium and potassium content of red cells did not exceed methodologic variability (see Table III).

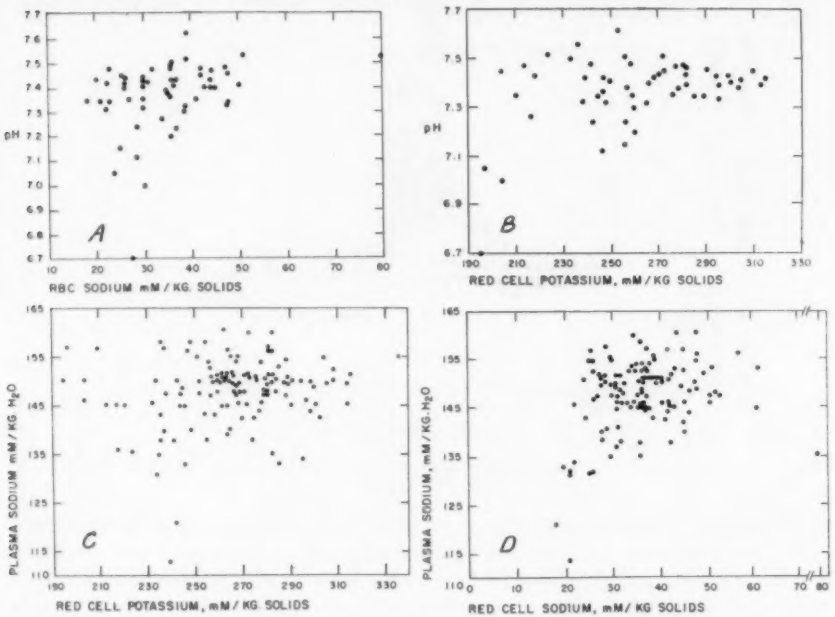


Fig. 4.—The relationship of the red cell sodium and potassium content to plasma pH, sodium, and potassium concentrations.

The data of all the patients, many with chronic illnesses, indicate that a wide range of red cell sodium values are found at low and high plasma sodium concentrations. At plasma sodium concentrations below 140 mM., however, low red cell sodium contents were observed rather frequently (see Fig. 4, *D*). No relationship is apparent between the red cell potassium content and the plasma sodium concentration (see Fig. 4, *C*).

2. The relationship of the red cell sodium, potassium, and water content to other plasma electrolytes: Fig. 4, *A* and *B*, shows that a low red cell sodium

*It has been stated that a large increase in the total body water content results in a large decrease in hematocrit and that opposite changes occur following water deprivation.[26] However, in the patient reported here, only a small drop in hematocrit occurred following a large change in total body water content, this decrease being accounted for by the difference in the water content of a liter of red cells and a liter of plasma.

and potassium content is occasionally observed in patients with alkalosis and acidosis. There is no relationship between the red cell potassium content and plasma potassium concentration, or between the plasma sodium concentration and the red cell water content (see Table I).

An elevation of the red cell sodium content more than 4 standard deviations above the normal mean value was observed in 5 patients. Vomiting had been present in 4 of these patients and diarrhea was present in the fifth. Alkalosis was probably present in 4 cases since the red cell to plasma chloride concentration ratio was reduced.[5] The red cell potassium content was normal in 4 of the patients with elevated red cell sodium content and markedly reduced in the fifth patient.

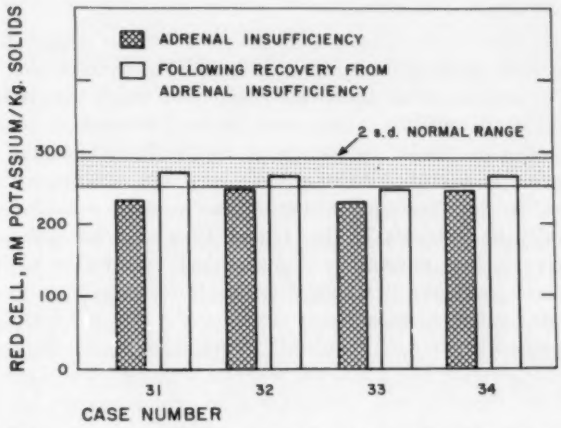


Fig. 5.—The red cell potassium content during adrenal insufficiency and after recovery.

C. Abnormalities of Red Cell Univalent Cation and Water Content in Adrenal, Renal, and Hepatic Insufficiencies.—

1. Adrenal insufficiency: In the 4 patients with acute adrenal insufficiency the red cell potassium content was reduced from 6 to 14 per cent below the mean normal content, and in 3 of the patients the red cell sodium content was also reduced (see Table II). Following recovery, the potassium content rose to the 95 per cent range of normal subjects in 3 instances and just below this range in the fourth patient (see Fig. 5). The red cell sodium content also rose in 3 instances (Table II).

2. Renal insufficiency: The nephritic patients with nitrogen retention and hyperkalemia frequently had low red cell sodium contents, usually in association with marked hyponatremia and acidosis (see Table I). The red cell potassium content was found to range from low to normal in these patients and it was not possible to identify a difference in the ionic composition of plasma which might explain the differing red cell potassium findings.

3. Hepatic insufficiency: Three of the 4 patients with Laennec's cirrhosis had a reduced red cell sodium and potassium content.

DISCUSSION

It had been reported by Darrow and Yannet that the concentration ratio of the univalent cations (sodium plus potassium) of red cells and plasma varied directly and equally when the osmotic pressure of the extracellular phase was acutely altered.[2] The alterations were not known to be associated with a change in pH. The findings of the present report, which includes data of patients with disturbances of hydrogen ion concentration, indicate that a direct and equal relationship of the concentration ratio of sodium plus potassium is present only if the diffusible anion concentration is included with the univalent cation concentration.

A decrease in the extracellular concentration of the dominant mineral solutes (sodium and chloride) requires, for osmotic equilibrium, that decreased content of solutes or an increased content of water occurs in the red cell. Knowledge of the plasma water, sodium, and potassium content does not provide a means of predicting which of these alterations has occurred, nor is the alteration of any one of the variables (water, sodium, or potassium contents) always in the direction which favors osmotic equilibrium. For example, chronically ill Subjects 1, 11, 14, and 19 with a low plasma sodium concentration have a low normal or reduced red cell water content (see Table I). In these patients the reduction in the red cell potassium and sodium content was sufficient to account for both the low red cell water content and the osmotic adjustment "required" by hyponatremia. Ten additional patients with hyponatremia had a normal red cell water content (see Table I). We would conclude that the red cell content of water cannot be predicted confidently from the knowledge of plasma composition in patients with chronic diseases.

The Significance of Changes in the Red Cell Water Content.—An elevation of the red cell water content may result from (1) a retention of water in the body, such as that produced by the administration of water and the hormone,[27] (2) an increase in cell solute content such as occurs in acidosis with a gain of water accompanying the chloride shift,[5] (3) a reduction in the extracellular solute concentration,[2] and (4) an increased proportion of young cells or cells with a low mean corpuscular hemoglobin concentration, as found in iron deficiency anemia.[28] Possibly several of the conditions affecting red cell water content were present in some of the chronically ill patients. In 11 patients with various diseases, elevated red cell water values were associated with an elevated red cell potassium content, normal plasma sodium concentration, and normal whole blood hemoglobin concentration. Possibly the accumulation of potassium and/or some organic anion (phosphates) was the event which resulted in gain of water.

The water content of red blood cells of the patients with the nephrotic syndrome and mild hyponatremia was normal. The data of Broch[29] and of

Fox, and Slobody[30] suggest that muscle cells also are not overhydrated during nephrotic edema.*

The Significance of Potassium Deficiency of Red Cells.—Red cells containing 6 to 25 per cent less potassium than normal have been found in conditions of potassium deficiency occurring in diabetic acidosis, gastric alkalosis, gastroenteritis, and base-losing nephritis.[7, 8, 12, 31-40] A similar percentage reduction of the total body potassium content, corrected for a change in nitrogen balance, has been observed in these diseases.[9-12, 18, 32, 34] In 4 patients with untreated adrenal insufficiency, in 5 of 11 with chronic nephritis and uremia, and in 3 of 4 with hepatic cirrhosis the red cell potassium content was more than two standard deviations below the average normal value. These patients were all seriously ill and eating little or no food. Hutt[8] has also reported low concentrations of potassium of red cells in patients with nephritis accompanied by hyperkalemia, and in one patient with Addison's disease.

The red cell data obtained from the patients with adrenal insufficiency are especially surprising since experimental animals with *acute*, surgically induced, adrenal insufficiency show an increase in muscle and red cell potassium content.[13-15] However, there are other data which support the suggestion that the low red cell potassium values found in chronic adrenal insufficiency may be associated with generalized cell potassium depletion. (1) Burrows and Sisson[41] have shown that the total body exchangeable potassium content per unit of body weight is below normal in some patients with Addison's disease†; and (2) Darrow[42] has reported that a group of adrenalectomized rats lost potassium "in excess of nitrogen" in association with anorexia and vomiting. Anorexia of long duration is often present in patients with adrenal insufficiency.[43]

Since the occurrence of reduced red cell potassium values has been reported only in patients with diseases known to be frequently associated with "generalized" cell potassium deficiency, it would appear to be more reasonable to assume that a total body cell potassium deficiency may be present in certain patients with chronic adrenal insufficiency than to assume that red cells are the only body cells that become depleted of potassium. Support for this thesis comes from the balance data of Talbot and Cook[44] which show retention of potassium in excess of nitrogen during the early treatment of adrenal insufficiency. The development in certain patients of weakness and low serum potassium concentrations by the second to the third day following the onset of treatment of adrenal insufficiency with DOCA and salt further suggests the possibility of low potassium reserves of body cells in these

*The data of Broch show that the muscle tissue of an edematous nephrotic subject contained a normal amount of nitrogen per kilogram of muscle. A chloride determination was not made; however, unless the ratio of nitrogen to muscle solids was elevated this datum suggests a normal muscle cell water content. By using the chloride method for partitioning muscle tissue water into extra- and intracellular phases, the data of Fox and Slobody show that the muscle cell water content of nephrotic patients is reduced.

†A low total body exchangeable potassium content per kilogram of body weight can result from an actual or a relative reduction in size of the cellular compartment, as well as from the loss of potassium "in excess of nitrogen" from cells. Hence, a low total body exchangeable potassium content does not constitute proof of cell potassium deficiency.

patients; it seems unlikely that potassium deficiency could have occurred so suddenly if the cells had contained a normal or increased amount of potassium before treatment was started.

The presence of hyperkalemia during the acute phase of adrenal insufficiency does not prejudice the thesis that low cell stores of potassium are present in some patients with chronic adrenal insufficiency since ketoacidotic patients with markedly reduced cell potassium contents often have hyperkalemia (10, 31, and 37); acidosis is often present in these patients as in patients with adrenal insufficiency.[43]

Evidence has been presented by Pitts and Alexander[45] and Berliner and co-workers[46] that the renal tubular exchange of hydrogen, and potassium for sodium, may involve competitive pathways. Since the human being with chronic adrenal insufficiency often eats large amounts of sodium chloride he has, in contrast to the acutely adrenalectomized animal, an opportunity to protect against immediate sodium depletion and circulatory collapse. However, he may not be able to conserve enough potassium to prevent potassium deficiency since anorexia and diarrhea frequently occur in chronically ill patients with Addison's disease.[43]

SUMMARY

The red cell *content** of water, sodium, potassium, and chloride was compared to the plasma composition of these substances in 103 patients with various diseases. It was found that the red cell content of sodium, and/or of potassium and water could not be predicted from plasma composition. The sodium plus potassium plus chloride *concentration*† of red cells was closely correlated to the plasma concentration of these electrolytes but the correlation of the sodium plus potassium concentration in the two fluid phases varied widely. Changes in the water content of red cells secondary to pH changes and the chloride shift appear to account for the poor correlation of the sodium plus potassium concentration of red cells and plasma.

An unexpected finding was the occurrence of a reduced red cell potassium content in 4 patients with adrenal insufficiency, in 5 of 11 patients with uremia and hyperkalemia, and in 3 of 4 with Laennec's cirrhosis. The discussion considers other data which suggest that generalized cell potassium deficiency is present in some human beings with chronic adrenal insufficiency.

The statistical analyses were carried out by Dr. Max Halperin, National Institutes of Health, Bethesda, Md.

REFERENCES

1. Guest, G. M.: Osmometric Behavior of Normal and Abnormal Human Erythrocytes, Blood **3**: 541, 1948.
2. Darrow, D. C., and Yannet, H.: The Changes in the Distribution of Body Water Accompanying Increase and Decrease in Extracellular Electrolyte, J. Clin. Invest. **14**: 266, 1935.
3. Christensen, H. N., and Riggs, T. R.: Physostigmine Uptake by Cells and Its Effect on Potassium Exchange, J. Biol. Chem. **193**: 621, 1951.

*Quantities per unit of solids.
†Quantities per unit of cell or plasma water.

4. Elkinton, J. R., Winkler, A. W., and Danowski, T. S.: Inactive Cell Base and Measurement of Changes in Cell Water, Yale J. Biol. & Med. **17**: 383, 1944.
5. Van Slyke, D. D., Wu, H., and McLean, F. C.: Studies of Gas and Electrolyte Equilibrium in the Blood, J. Biol. Chem. **56**: 765, 1923.
6. Dawson, J., Dempsey, E., Bartter, F., Leaf, A., and Albright, F.: Evidence for the Presence of an Amphoteric Electrolyte in the Urine of Patients With "Renal Tubular Acidosis," Metabolism **2**: 225, 1953.
7. Danowski, T. S., Hald, P. M., and Peters, J. P.: Sodium, Potassium and Phosphates in the Cells and Serum of Blood in Diabetic Acidosis, Am. J. Physiol. **149**: 667, 1947.
8. Hutt, M. P.: Effect of Disease on Erythrocyte and Plasma Potassium Concentrations, Am. J. M. Sc. **223**: 176, 1952.
9. Atchley, D. W., Loeb, R. F., Richards, D. W., Jr., Benedict, E. M., and Driscoll, M. E.: On Diabetic Acidosis; A Detailed Study of Electrolyte Balances Following the Withdrawal and Reestablishment of Insulin Therapy, J. Clin. Invest. **12**: 297, 1933.
10. Danowski, T. S., Peters, J. H., Rathbun, J. C., Quashnock, J. M., and Greenman, L.: Studies in Diabetic Acidosis and Coma, With Particular Emphasis on the Retention of Administered Potassium, J. Clin. Invest. **28**: 1, 1949.
11. Butler, A. M.: Diabetic Coma, New England J. Med. **243**: 648, 1950.
12. Wyngaarden, J. B., Keitel, H. G., and Isselbacher, K. J.: Potassium Depletion and Alkalosis. Their Association With Hypertension and Renal Insufficiency, New England J. Med. **250**: 597, 1954.
13. Darrow, D. C., Harrison, H. E., and Taffel, M.: Tissue Electrolytes in Adrenal Insufficiency, J. Biol. Chem. **130**: 487, 1939.
14. Harrison, H. E., and Darrow, D. C.: The Distribution of Body Water and Electrolytes in Adrenal Insufficiency, J. Clin. Invest. **17**: 77, 1938.
15. Muntwyler, E., Mellors, R. C., Mautz, F. R., and Mangun, G. H.: Electrolyte and Water Equilibria in the Dog: Electrolyte and Water Exchange Between Skeletal Muscle and Blood in Adrenal Insufficiency, J. Clin. Invest. **134**: 367, 1940.
16. Elkinton, J. R., and Danowski, T. S.: The Body Fluids, Baltimore, 1955, Williams & Wilkins Company, 1955.
17. Gribetz, D., Corsa, L., Jr., Cook, C. D., Keitel, H. G., and Talbot, N. B.: Measurement of Total Body Exchangeable Potassium and Erythrocyte Potassium in Nephrotic Children, J. Clin. Invest. **33**: 680, 1954.
18. Keitel, H. G., and Jones, H.: Comparison of Serial Change in Red Cell Potassium Content With Total Body Potassium Balance During Potassium Therapy, J. LAB. & CLIN. MED. (In press.)
19. Keitel, H. G., Berman, H., Jones, H., and MacLachlan, E.: The Chemical Composition of Normal Human Red Blood Cells Including Variability Among Centrifuged Cells, Blood **10**: 370, 1955.
20. Berry, J. W., Chappell, D. G., and Barnes, R. B.: Improved Method of Flame Photometry, Indust. & Engin. Chem. (Anal. Ed.) **18**: 19, 1946.
21. Volhard, J.: Die Silbertigrirung mit schwefelcyanammonium, Ztschr. anal. Chem. **17**: 482, 1878.
22. Wilson, D. W., and Ball, E. G.: A Study of the Estimation of Chloride in Blood and Serum, J. Biol. Chem. **79**: 221, 1928.
23. Van Slyke, D. D., and Neill, J. M.: The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurement, J. Biol. Chem. **61**: 523, 1924.
24. Ponder, E.: Hemolysis and Related Phenomena, New York, 1948, Grune & Stratton, Inc., p. 115.
25. Allen, F. M., and Sherrill, J. W.: Diet Treatment of Diabetes Insipidus, J. Metab. Res. **3**: 479, 1923.
26. Dancis, J., Birmingham, J. R., and Leslie, S. H.: Congenital Diabetes Insipidus Resistant to Treatment With Pitressin, Am. J. Dis. Child. **69**: 323, 1945.
27. Unpublished observations.
28. Maizels, M.: The Anion and Cation Contents of Normal and Anaemic Bloods, Biochem. J. **30**: 821, 1936.
29. Broch, O. J.: Serum Protein and Tissue Protein With Special Reference to Conditions of Protein Depletion, Acta med. scandinav. **141**: 6, 1951.
30. Fox, C. L., Jr., and Slobody, L. B.: Tissue Changes in the Nephrotic Syndrome: Demonstration of Potassium Depletion, Pediatrics **7**: 186, 1951.
31. Guest, G. M., and Rapoport, S.: Electrolytes of Blood Plasma and Cells in Diabetic Acidosis and During Recovery, Proc. Am. Diabetes A. **7**: 95, 1947.
32. Kennedy, T. J., Jr., Winkley, J. H., and Dunning, M. F.: Gastric Alkalosis With Hypokalemia, Am. J. Med. **6**: 790, 1949.

33. Lans, H. S., Stein, I. F., and Meyer, K. A.: The Relation of Serum Potassium to Erythrocyte Potassium in Normal Subjects and Patients With Potassium Deficiency, Am. J. M. Sc. **223**: 65, 1952.
34. Eliel, L. P., and Pearson, O. H.: The Metabolic Effects of Adrenocorticotrophic Hormone (ACTH) in a Patient With Cushing's Syndrome and Acromegaly, J. Clin. Endocrinol. **11**: 913, 1951.
35. Kauhtio, J., and Hallman, N.: Sodium and Potassium Concentration of Erythrocytes and Bromsulphalein Liver Function Test in Severe Infantile Gastroenteritis, Acta paediat. **39**: 328, 1950.
36. Ettledorf, J. N., Hill, F. S., Tuttle, A. H., Pinheiro, D., Bass, A. G., and Overman, R. R.: Alterations in Blood Chemistry Caused by Diarrhea During Infancy as Determined by Direct Analysis of Plasma and Erythrocytes, Pediatrics **10**: 694, 1952.
37. Nichols, G., Jr., and Nichols, N.: Electrolyte Equilibria in Erythrocytes During Diabetic Acidosis, J. Clin. Invest. **32**: 113, 1953.
38. Eliel, L. P., Pearson, O. H., and White, F. C.: Postoperative Potassium Deficit and Metabolic Alkalosis. The Pathogenic Significance of Operative Trauma and of Potassium and Phosphorus Deprivation, J. Clin. Invest. **31**: 419, 1952.
39. Eliel, L. P., Pearson, O. H., and Rawson, R. W.: Postoperative Potassium Deficit and Metabolic Alkalosis, New England J. Med. **243**: 471, 1950.
40. Hoffman, W. S., and Jacobs, H. R. D.: The Partition of Potassium Between the Serum and Corpuscles in Health and Disease, J. LAB. & CLIN. MED. **19**: 633, 1934.
41. Burrows, B. A., and Sisson, J. H.: The Measurement of Total Body Potassium by the Radioisotope Dilution Technique, J. Clin. Invest. **29**: 801, 1950.
42. Darrow, D. C.: Tissue Water and Electrolyte, Am. Rev. Physiol. **6**: 95, 1944.
43. Thorn, G. W., Forsham, P. H., and Emerson, K., Jr.: The Diagnosis and Treatment of Adrenal Insufficiency, Springfield, Ill., 1949, Charles C Thomas, Publisher, p. 31.
44. Personal communication to the authors.
45. Pitts, R. F., and Alexander, R. S.: The Nature of the Renal Tubular Mechanism for Acidifying the Urine, Am. J. Physiol. **144**: 239, 1945.
46. Berliner, R. W., Kennedy, T. J., Jr., and Orloff, J.: Relationship Between Acidification of the Urine and Potassium Metabolism: Effect of Carbonic Anhydrase Inhibition on Potassium Excretion, Am. J. Med. **11**: 274, 1951.

URINARY SPECIFIC GRAVITY VERSUS TOTAL SOLUTE CONCENTRATION: A CRITICAL COMPARISON

I. Studies in Normal Adults

Edgar J. Schoen, M.D., George Young, A.B., and Arthur Weissman, J.D.
Oakland, Calif.

THE estimation of urinary specific gravity has long been a medical diagnostic tool; its measurement under conditions of water deprivation and water loading (the Fishberg dilution-concentration test) is widely accepted as an indication of the parameters of renal concentration and dilution of solutes. The term "specific gravity," in medical usage, refers to the density of a solution as compared with that of pure water. It is usually measured by means of a hydrometer calibrated to give a reading of 1.000 when floated in distilled water at 15.6° C.

Specific gravity gives no information about the number of molecules in solution. Osmolarity (usually measured by the vapor pressure of freezing point depression method) represents the total number of molecules present in solution, regardless of the molecular weight or other physical properties of the individual solutes.

Various observers[1-3] have indicated that a more accurate estimate of the renal mechanisms involved in solute concentration can be made on the basis of urinary osmolarity than by determination of the specific gravity. Addis and Foster[4] emphasized that the estimate of urinary total solids from specific gravity measurements lacks quantitative significance. Price and associates[5] showed that an equal number of milliequivalents of each solute in the urine contributes to the specific gravity in a different measure, the total specific gravity representing only the sum of the individual specific gravities. Miles and his co-workers,[2] in comparing specific gravity and osmolarity, noted that "specific gravity is only a rough indication of osmolarity even in normal urines." These authors observed that the discrepancy is greatest at higher concentrations, but did not express correlation values between specific gravity and osmolarity at various concentrations. The measurement of specific gravity thus appears to be of clinical value only as it reflects urinary osmolarity.

In the observations presented here, the specific gravity and total solute concentration of the urine of normal subjects were measured and compared over the range of dilution and concentration.

From the Department of Pediatrics, Permanente Medical Group, Kaiser Foundation Hospital, and the Office of Statistical Information, Kaiser Services, Oakland, Calif.
This investigation was supported by a grant from the Kaiser Foundation.
Received for publication Dec. 15, 1958.

MATERIALS AND METHODS

The subjects were 10 normal men and 2 normal women, 25 to 35 years old. From each subject, voided and timed urine specimens were collected in separate aliquots over a continuous 2-day period. The urines were shown by qualitative test to be free of glucose and protein. Specific gravity and osmolarity were determined within 24 hours of collection. When, as for specimens voided during the evening hours, determinations could not be performed immediately, the urine was refrigerated overnight. The maximally diluted specimens were collected under the conditions of water loading previously described.[3] Concentrated urine specimens were collected according to the technique described by Frank and co-workers[1] which includes withholding of fluids for 30 hours. Three hundred and thirty-three urine specimens were studied.

Urinary specific gravity was measured with both a standard ward hydrometer, calibrated at 15.6° C., and a Westphal balance (pyknometer), calibrated at 4° and 20° C. All measurements of specific gravity were made at 20° C. and 0.001 was added to each hydrometer reading to correct for the 4.4° variation above 15.6° C.[2] Urinary total solute concentration was determined by the freezing point depression method, using the Fiske osmometer.

Specific gravity values in increments of 0.003 from 1.000 to 1.032 were plotted against urinary osmolarity values; single points were plotted at sp. gr. 1.034 and 1.038.

RESULTS

There was a high degree of correlation (coefficient of correlation 0.97) between the urinary osmolarity and specific gravity at all levels of urinary solute concentration (Fig. 1). The degree of correlation was highest in the high and the low specific gravity ranges; whereas the specific gravity values tended to decrease per increment of rise in osmolarity in the middle ranges of specific gravity (sp. gr. 1.010 to 1.020).

Frank and his co-workers[1] considered a urinary total solute concentration of 800 mOsm. per liter or greater to represent "excellent" renal concentrating ability. In the cases reported here readings above 800 mOsm. per liter were found in 1 of 23 values in the 1.012 to 1.014 range, in about one-third (6 of 20) of the specimens showing a specific gravity between 1.015 and 1.017, in two-thirds of those with specific gravity ranging from 1.018 to 1.020, and in almost all with specific gravity between 1.021 and 1.023 (among the 43 specimens in the last group were two with values below 800 mOsm. per liter: 773 and 786 mOsm., respectively). The osmolarity was greater than 900 mOsm. per liter in all instances in which the specific gravity was greater than 1.023.

The minimum urinary concentration (osmolarity below 80 mOsm. per liter)[3, 6] was represented by specific gravity values lower than 1.005 in all instances.

Urinary isosmolarity (the osmolar level at which urine and serum concentrations are equal) is represented by a value approximating 300 mOsm. per liter. The urinary specific gravity levels most closely associated with isosmolarity were between 1.006 and 1.008 (Table I) (mean osmolarity, 269; standard deviation, 93). The specific gravity that is commonly considered to represent urinary isosmolarity, 1.009 to 1.011, was found in specimens with higher osmolarity levels (mean osmolarity reading, 424 mOsm. per liter;

standard deviation, 68). The osmolarity of 23 of the 24 specimens having a specific gravity of 1.010 was greater than 320 mOsm. per liter.

Although the over-all correlation between urinary specific gravity and osmolarity was high in these normal adults, a high osmolarity level was found to be associated with a low specific gravity reading in some cases. Examples were: specific gravity 1.003, osmolarity 262; specific gravity 1.010, osmolarity 686; specific gravity 1.012, osmolarity 972; 7 instances in which osmolarity was greater than 800 mOsm. per liter whereas specific gravity was below 1.017.

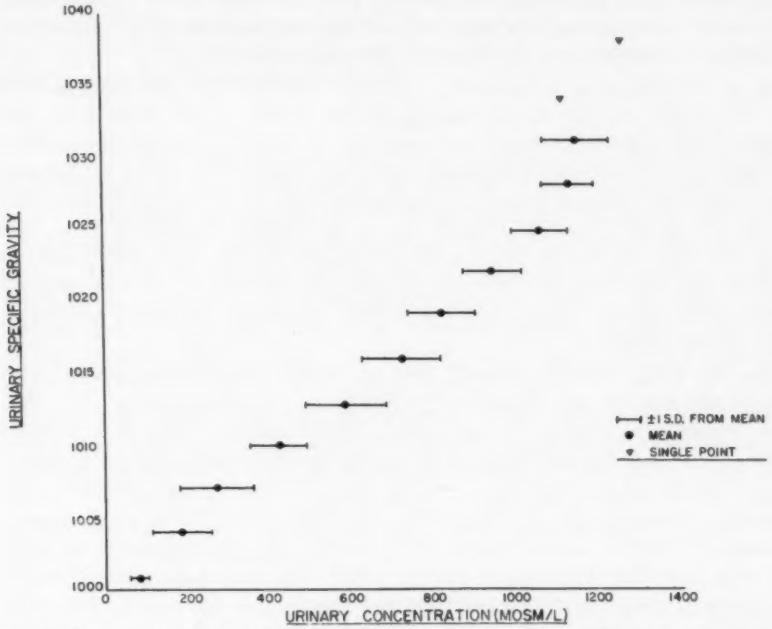


Fig. 1.—Means and standard deviations of urinary osmolarity plotted against urinary specific gravity levels at intervals of 0.003.

TABLE I. MEANS, STANDARD DEVIATIONS, AND RANGES OF URINARY OSMOLARITY BY SPECIFIC GRAVITY

SPECIFIC GRAVITY	NUMBER	MEAN (MOSM./L.)	STANDARD DEVIATION (MOSM./L.)	RANGE OF OSMOLARITY (MOSM./L.)
1.000-1.002	65	78	24	52- 193
1.003-1.005	40	180	75	61- 367
1.006-1.008	28	269	93	106- 473
1.009-1.011	39	424	68	249- 584
1.012-1.014	23	582	98	439- 972
1.015-1.017	20	721	94	533- 825
1.018-1.020	24	816	82	600- 944
1.021-1.023	43	944	73	773-1,085
1.024-1.026	23	1,060	63	955-1,226
1.027-1.029	15	1,125	67	1,012-1,262
1.030-1.032	11	1,147	81	990-1,282
1.034	1	1,110	–	–
1.038	1	1,262	–	–

In determining urinary specific gravity at constant temperature, the readings observed on a tested clinical hydrometer were found to be accurate when compared with the readings of specific gravity on a Westphal balance.

DISCUSSION

According to our observations in normal adults, a specific gravity reading in the higher ranges (above 1.020) may be said to represent an osmotically concentrated urine if osmolarity above 800 mOsm. per liter is accepted as a valid indication of adequate urinary concentration. But urinary specific gravity lower than 1.020 does not indicate that the urine is not concentrated. The urine of some normal subjects had an osmolarity greater than 800 mOsm. per liter and a specific gravity between 1.012 and 1.017.

It is hazardous to estimate the osmolar concentration of urine on the basis of specific gravity in the middle or low range. Urine with a specific gravity below 1.005 is probably hypo-osmolar to serum. The specific gravity of isosmolar urine is usually between 1.006 and 1.008. A reading of 1.010 or higher usually represents hyperosmolarity, and the mean osmolarity increases with rising specific gravity (Table I). If a definite osmolarity (e.g., 800 mOsm. per liter or above) is considered the criterion of "adequate" urinary concentration, one cannot estimate whether an individual specific gravity reading in the middle range (1.010 to 1.020) represents "adequate" or inadequate" concentration.

The currently utilized clinical test of renal concentrating ability[7] employs specific gravity as a measure of urinary solute concentration. The subject is considered to have normal renal concentrating ability if a urinary specific gravity reading of 1.022 or above is obtained following a period of thirsting of 24 to 30 hours. Our data confirm the validity of specific gravity as an expression of osmotic concentration when the reading is 1.022 or above in normal subjects, but the osmotic concentration may be equally high when the specific gravity is within the range of 1.012 to 1.022. The false impression of inadequate concentrating ability gained from the specific gravity reading may lead to unnecessary repetition or prolongation of the test.

It has been shown that both glucose and protein raise specific gravity more than they increase osmolarity.[2] The presence of protein and to a small extent the presence of glucose in the serum account for the fact that a serum osmolarity of 300 mOsm. per liter is associated with a specific gravity of 1.010, whereas urine with an osmolarity of 300 mOsm. per liter usually has a specific gravity within the range of 1.006 to 1.008. We have observed urine specimens containing large quantities of glucose, in which a specific gravity greater than 1.030 was associated with an osmolarity of less than 800 mOsm. per liter. Thus, when abnormal or unusual solutes are present in the urine, a high specific gravity reading may not represent intense osmotic concentration. If specific gravity is to be used as an indicator of urinary total solute concentration within the higher range in normal subjects, the urine should be shown to be free of sugar or protein.

The adoption of the direct measurement of urinary osmolarity for clinical work would permit comparison of urinary total solute concentration with that of other body fluids, and with the concentration of individual solutes in urine. It would further permit the establishment of new quantitative standards for urinary solute dilution and concentration, making possible a more accurate estimate of deviations from these values in renal disease.

Urinary osmolarity (and the osmolarity of other body fluids as well) is easily determined with currently available equipment to an accuracy within plus or minus 3 mOsm. per liter. Techniques are available for measuring total osmolarity in renal micropuncture studies[8] and in other very small quantities of fluid.[9] The clinical measurement of osmolarity is simple and rapid. Modern "osmometers" have a scale permitting the direct reading of osmolarity once the machine is standardized.

SUMMARY

The measurement of urinary specific gravity as an indication of renal concentrating and diluting ability is valid only as it reflects urinary total solute concentration (osmolarity). In normal subjects having no evidence of glycosuria or proteinuria, the urinary specific gravity in the high range (above 1.020) reflects well the urinary osmolarity and represents a urinary total solute concentration above 800 mOsm. per liter. A urinary specific gravity below 1.005 may be said to represent a hypo-osmolar urine. In the range between 1.005 and 1.020, the specific gravity reading may be misleading as an indication of urinary total solute concentration.

The advantages of direct measurement of urinary osmolarity for clinical work are: (1) it is more accurate than the specific gravity measurement made by currently available equipment; (2) it permits finer quantitation; (3) it lessens the need for prolonged thirsting and similar devices; and (4) it permits direct comparison of concentrations of total and individual solutes with those of other body fluids.

REFERENCES

1. Frank, M. N., Dreifus, L. S., Rarick, F., and Bellet, S.: Urinary Osmolar Concentration in the Hydropenic State as a Measure of Renal Tubular Function: A Test for Early Renal Impairment: Preliminary Report, Am. J. M. Sc. **233**: 121-125, 1957.
2. Miles, B. E., Paton, A., and de Wardener, H. E.: Maximum Urine Concentration, Brit. M. J. **2**: 901-905, 1954.
3. Schoen, E. J.: Minimum Urine Total Solute Concentration in Response to Water Loading in Normal Men, J. Appl. Physiol. **10**: 267-270, 1957.
4. Addis, T., and Foster, M. G.: The Specific Gravity of the Urine, A. M. A. Arch. Int. Med. **30**: 555-558, 1922.
5. Price, J. W., Miller, M., and Hayman, J. M., Jr.: The Relation of Specific Gravity to Composition and Total Solids in Normal Human Urine, J. Clin. Invest. **19**: 537-554, 1940.
6. Kleeman, C. R., Epstein, F. H., and White, C.: The Effect of Variations in Solute Excretion and Glomerular Filtration on Water Diuresis, J. Clin. Invest. **35**: 749-756, 1956.
7. Fishberg, A. M.: Hypertension and Nephritis, ed. 5, Philadelphia, 1954, Lea & Febiger.
8. Hargitay, B., Kuhn, W., and Wirz, H.: Eine mikrokryoskopische Methode für sehr kleine Lösungsmengen, Experientia (Basel) **7**: 276-278, 1951.
9. Ramsay, J. A.: A New Method of Freezing-point Determination for Small Quantities, J. Exper. Biol. **26**: 57-64, 1949.

LABORATORY METHODS

THE USE OF THE FLUORESCENT ANTIBODY TECHNIQUE FOR THE DEMONSTRATION OF ERYTHROCYTE ANTIGENS

JoAnne Whitaker, M.D., Wolf W. Zuelzer, M.D., Abner R. Robinson, M.S., and Margaret Evans, M.S.
Detroit, Mich.

A VARIETY of specific antigen-antibody reactions involving tissues, microorganisms, and viruses have been readily demonstrated by means of the fluorescent antibody technique introduced by Coons and Kaplan.[1] The visualization of antigen-antibody systems involving antigenic components of the erythrocyte by this method has been unsuccessful to date. To our knowledge, no reports on this subject have appeared in the literature despite its obvious theoretical and potential importance. We wish to report studies in which the A and B antigens in erythrocytes were visualized by fluorescent antibodies.

Hyperimmune human anti-A and anti-B sera, obtained from volunteer donors injected with commercial A and B substances were precipitated with 17.5 per cent $NaSO_4$, and the gamma globulin fractions thus obtained (hereafter designated as antisera) were coupled with fluorescein isocyanate by the Coons-Goldman paper technique.[2] After coupling, the anti-A and anti-B titers were 1:256. After allowing the fluorescein-labeled preparation to react with erythrocytes from groups A, B, and O, the latter were observed under a Reichert microscope with a mercury arc lamp with appropriate filters under dark-field illumination.

In our preliminary studies we were unable to demonstrate fluorescence of group A erythrocytes, even though the cells were agglutinated by the appropriate sera and the antibodies could be eluted from the cells or from stroma preparations. The eluted material was again fluorescent under a Wood's lamp and agglutinated erythrocytes of proper specificity. In view of these findings we assumed that failure to observe fluorescence was due to a "quenching" effect by factors in the erythrocyte rather than in insufficient receptor sites.

A quenching effect on the fluorescence of labeled antiserum was demonstratable by observing the material under a Wood's lamp upon addition of a variety of oxidizing and reducing agents. Complete and immediate quenching was noted after addition of agents known to destroy sulfhydryl linkages. The addition of hemoglobin, however, did not produce quenching although there was a slight diminution of the intensity of fluorescence. Other factors in the erythrocytes were thought, therefore, to be responsible.

From the Child Research Center of Michigan and The Department of Pediatrics, Wayne State University College of Medicine, Detroit, Mich.

Supported by United States Public Health Service Grant No. H-3119.

Read before the Society for Pediatric Research, 28th Annual Meeting, Atlantic City, May 6-7, 1958.

Received for publication July 15, 1958.

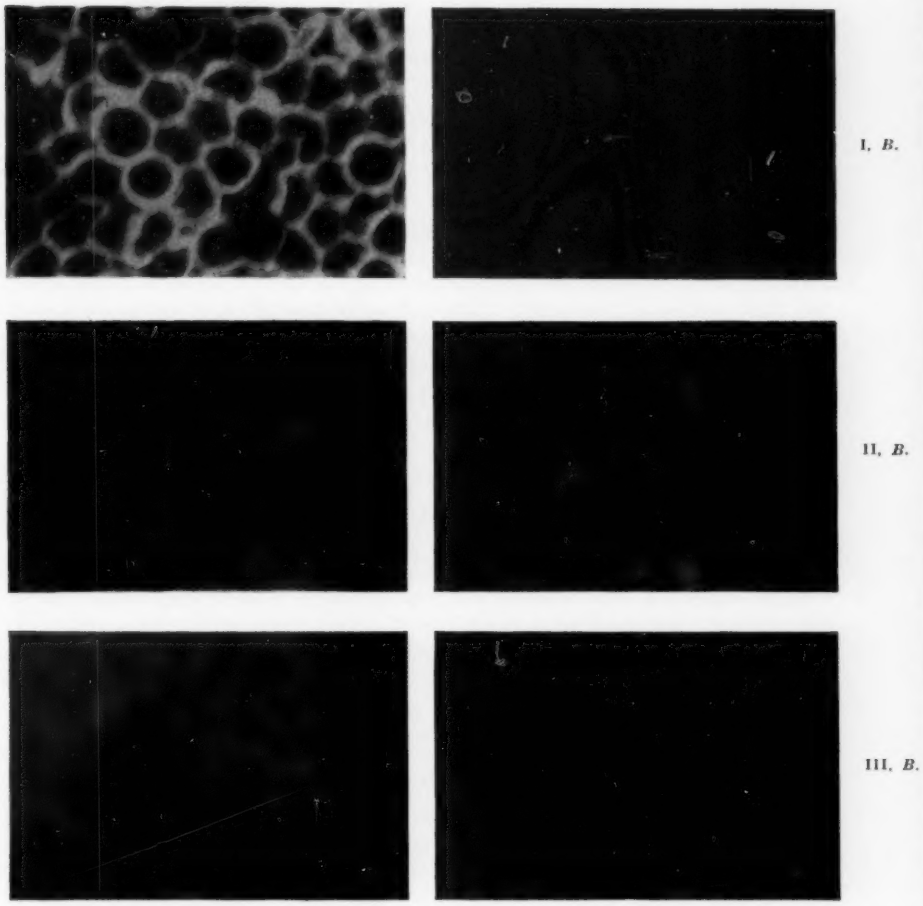


Fig. 1.—

I. Controls: Group A erythrocytes untreated, regular.
A, Regular dark-field illumination.
B, Fluorescent light.

II. Group A erythrocytes: Versene-treated and stained with hyperimmune labeled anti-A antiserum viewed with fluorescent light.
A, Serum absorbed with group 0 stroma.
B, Serum absorbed with mouse liver powder.

III. *A*, Serum absorbed with group B stroma.
B, Serum absorbed with group A stroma.

Since it is known that heavy metals present in erythrocytes can quench resonance, a number of chelating agents, among them Versene (EDTA), BAL, and 8-oxyquinoline, were investigated in an attempt to neutralize the quenching effect. Red blood cells were washed 3 times in normal saline. One milliliter of packed cells was allowed to stand for 30 minutes with 10 mg. of dry Versene. The cells were again washed with normal saline. Labeled antiserum (0.2 ml.) was mixed on an agglutination slide with 0.2 ml. of packed red blood cells and the slide shaken for 45 minutes. The cells were again washed 3 times in normal saline, suspended in 10 per cent buffered glycerine, and mounted.

By pretreatment with Versene, BAL, and 8-oxyquinoline we were able to demonstrate specific fluorescent membranes of group A cells stained with the same anti-A preparation which had otherwise given consistently negative results (Fig. 1). The specificity of the staining was documented by preabsorbing the labeled antiserum with mouse liver powder and with erythrocytes and stroma from group A, B, and O individuals. A-absorbed antiserum had lost its ability to agglutinate or render fluorescent group A erythrocytes. B- and O-absorbed antiserum remained effective. B and O erythrocytes showed no fluorescence. Versene-treated A_2 cells showed faint fluorescent membranes.

Group B erythrocytes, treated with the particular labeled anti-B used were found to show fluorescent membranes even without pretreatment with Versene, but, after the chelating agent and mechanical agitation were used, fluorescence was consistently enhanced and single fluorescent erythrocyte membranes were more distinct.

Attempts to demonstrate Rh (anti-D) reactions with high titered labeled anti-D saline and albumin type antisera were consistently unsuccessful, even when chelating agents were used. It was noted that treatment with Versene diminished or abolished the direct Coombs' antiglobulin test, whereas addition of Versene to originally unsensitized red blood cells had no effect on the indirect Coombs' test. This suggested that the Versene must have some effect on the antibody coating layered on the red blood cells.

SUMMARY

It has been shown that pretreatment of the red blood cells with chelating agents and agitation during the staining process intensifies the specific fluorescence of the erythrocyte membrane. Versene diminishes or abolishes the Coombs' test on sensitized cells, but has no effect on unsensitized cells.

REFERENCES

1. Coons, A. H., and Kaplan, M. H.: Localization of Antigen in Tissue Cells. II. Improvements in a Method for the Detection of Antigen by Means of Fluorescent Antibody, J. Exper. Med. **91**: 1-13, 1950.
2. Goldman, M., and Carver, R. K.: Preserving Fluorescein Isocyanate for Simplified Preparation of Fluorescent Antibodies, Science **126**: 839-40, 1957.

MEASUREMENT OF PROTEOLYTIC ACTIVITY OF BLOOD WITH A SUBSTRATE TAGGED WITH RADIOACTIVE IODINE

J. C. Heuson, M.D.*
Brussels, Belgium

FIBRINOLYTIC activity is occasionally found in the plasma of patients with a variety of pathologic conditions. Its presence is often detected by observing the complete dissolution of the clot of fibrin formed by the coagulation of blood.[1] The nature of the enzyme or enzymes responsible for this activity of blood in disease and their relation to the enzyme plasmin have been under discussion but do not constitute the subject of this paper. Our purpose is to describe a method by which the proteolytic activity of blood plasma can be accurately detected and quantitatively determined; the method which is based on the use of casein tagged with radioactive iodine and the measurement of radioactive products of the reaction offers advantages over the methods used so far for the detection and evaluation of clinical fibrinolysis. The data presented here show that iodinated casein is an adequate substrate for the measurement of proteolytic activity and results obtained with plasmin will be given here. Data presented elsewhere[4] show that the same substrate can be used with blood serum from cases of clinical fibrinolysis for the measurement of the proteolytic activity observed in cases of clinical fibrinolysis.

MATERIALS AND METHODS

Reagents.—

Plasmin: Fraction $III_{2,1}$ from human plasma prepared by the method of Cohn. (Obtained through the courtesy of Dr. J. T. Edsall.)

Buffer: Tris buffer, 0.05 M, adjusted to pH 7.7 by the addition of hydrochloric acid.

Ion exchange resin: Amberlite† IRA 410 in its alkaline form was used.

Trichloroacetic acid: One part of a 6 per cent solution was added to 1 part of the protein solution for precipitation purposes.

Casein: Twelve grams of casein‡ was added with stirring to 150 ml. of distilled water. The pH was adjusted to 6.0 by the addition of 0.5 N sodium hydroxide. The suspension was heated to 60° C. for 5 minutes to bring about complete dissolution. After cooling, the solution was filtered through Whatman No. 4 paper.

Iodination of casein: The radioactive iodine used was I^{131} with a half-life of 8 days obtained as a carrier-free sodium iodide solution in approximately 0.01 N sodium thiosulfate. It was diluted with water to a concentration of 1.5 mc. of I^{131} per milliliter.

From the Department of Medicine and Clinical Investigation, Institut Jules Bordet, Centre anticancéreux de l'Université de Bruxelles, Belgium.
Received for publication Dec. 19, 1958.

*Fellow of the Institut Interuniversitaire des Sciences Nucléaires, Brussels, Belgium.
†Supplied by Rohm & Haas, Philadelphia, Pa.
‡Hammarsten, Merck & Company, Inc., Rahway, N. J.

The following reagents were added to the solution of casein: 1.5 ml. of 0.1 M potassium iodide, 2.5 mc. of radioactive iodine and then, drop by drop with stirring, 1.5 ml. of a 30 per cent solution of hydrogen peroxide. The mixture was incubated at 40° C. for 60 minutes, at which time over 99 per cent of the radioactivity was bound to the casein and could be precipitated by trichloroacetic acid.

Twenty-five milliliters of Amberlite was added to the mixture. After stirring for 15 minutes, it was filtered through Whatman No. 4 paper. The pH was adjusted to 7.3 by the addition of 0.5 N sodium hydroxide and then 5 ml. of 0.1 M potassium iodide were added. The solution was heated to 90° C. for 15 minutes and cooled to room temperature. Twenty-five milliliters of Amberlite was added, the mixture was stirred for 15 minutes and filtered through Whatman No. 4 paper.

The protein concentration in the resulting solution was adjusted to approximately 2 per cent by the addition of distilled water and the pH brought to 7.7 by the addition of 0.5 N NaOH. This constituted the substrate and it was stored in small lots at -20° C. until used.

Properties of the Iodinated Casein.—Less than 0.5 per cent of the total radioactivity of the casein solution was found in the soluble fraction after precipitation by trichloroacetic acid. This soluble radioactivity was not increased by heating the substrate at 37° C. for at least one hour.

The iodinated substrate could be used for a period of two months following its preparation.

Estimation of Casein Concentration.—This was done by measuring the optical density at 280 mμ in a Beckman DU spectrophotometer. The extinction coefficient of iodocasein in aqueous solution was calculated from total nitrogen determinations.

Optical density readings were also made on the trichloroacetic acid filtrates obtained after proteolysis of the casein solution as a measure of enzyme action. The same extinction coefficient was used[3] and it was found that shifting the pH from 7.7 to 1.2 did not affect the readings.

Determination of Proteolytic Activity.—The reaction was carried out at 37° C. in test tubes immersed in a constant temperature water bath. One milliliter of the enzyme solution was added to 1 ml. of the substrate. After 60 minutes' incubation, the reaction was stopped by the addition of 2 ml. of trichloroacetic acid. After an hour at room temperature, the precipitate was filtered through Green No. 803 paper. The radioactivity of the filtrate was measured on a 1 or 2 ml. aliquot in a well-type scintillation counter. The increase in radioactivity resulting from the enzyme action was calculated by subtracting the radioactivity of a blank. The blank was obtained by incubating separately the substrate and the enzyme solution and by mixing them at the end of the incubation time, immediately before the addition of the trichloroacetic acid. Results were expressed as milligrams of digested casein and were calculated by dividing the increase in radioactivity by the specific radioactivity of the iodocasein.

RESULTS

Plasmin Activity as Measured by Radioactivity of Trichloroacetic Acid Filtrate.—Six determinations were carried out with 0.5 mg. of plasmin in 1 ml. of buffer. The mean increase in radioactivity of the trichloroacetic acid filtrates was measured and found to correspond to 0.476 mg. of digested casein ($\sigma = 0.022$). In the same filtrates, the mean increase in optical density at 280 mμ corresponded to 0.452 mg. of digested casein ($\sigma = 0.041$). These results do not differ significantly ($t = 1.25$).

In a series of 12 determinations carried out simultaneously with 0.5 mg. of plasmin in 1 ml. of buffer, the variation coefficient among the results was found to be 2.6 per cent of the mean value.

In another experiment, varying amounts of plasmin were incubated with a constant amount of substrate and the radioactivity in the trichloroacetic acid filtrates of each sample was measured. The increase in radioactivity was found to be proportional to the enzyme concentration. The standard curve for estimation of plasmin within this range of concentrations was a straight line (Fig. 1).

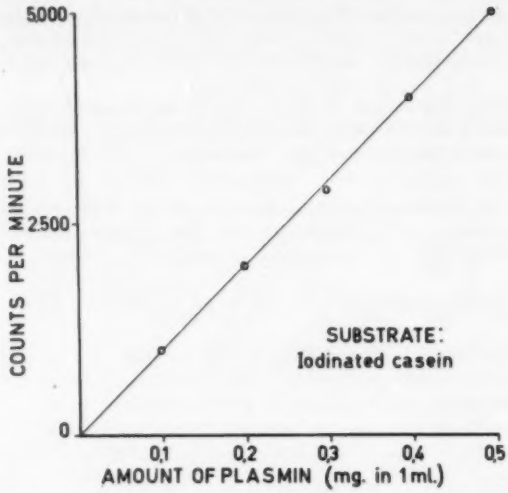


Fig. 1.—Proteolysis of tagged casein by plasmin as measured by the increase in radioactivity of the trichloroacetic acid filtrate. Reaction carried out at 37° C. and pH 7.7. Incubation time was 60 minutes. Values given are averages of duplicate determinations.

As far as the effect of the addition of serum to iodinated casein on the value of the blank is concerned, it was found that the blank was not increased by substituting serum for buffer solution in the enzyme experiments. In contrast, this substitution of serum greatly increased the optical density of the mixture. These observations are summarized in Table I which gives the value of the blank obtained when fresh serum is present for both the radioactive and optical density methods of measurement. With the radioactive method, the blank appears to be independent of the reagents present in the mixture and this was shown to hold true also for two other reagents added to the reaction mixture and which were tested because they are often used in enzyme experiments (Table I).

DISCUSSION

Radioactive iodinated casein was easily prepared by the method described here and could be used for at least two months when stored in the cold. The data indicate that increases of radioactivity, as measured by gamma ray counts, in the supernatant solution from a trichloroacetic acid precipitation can be taken as a measure of enzyme activity. The measurements by this method were simple and reproducible. The amount of radioactivity in the tagged substrate was small and did not constitute a health hazard if a few simple precautions were taken.

TABLE I. EFFECT OF THE ADDITION OF CERTAIN REAGENTS TO THE REACTION MIXTURE ON THE VALUE OF THE BLANK MEASURED BY EITHER RADIOACTIVITY OR OPTICAL DENSITY

REAGENTS ADDED*	BLANK VALUES†	
	BY RADIOACTIVITY	BY OPTICAL DENSITY
Serum	0.93	16.2
ATP (0.015 M)	1.12	infinite
Nicotinamide (0.03 M)	1.02	infinite

*In the reaction mixture, the buffer solution was replaced by the reagent in identical amount.

†Expressed as multiples of the value obtained with buffer instead of the reagent.

Although this method can probably be applied to other proteolytic enzymes, it appears especially advantageous for the study of the proteolytic activity of blood encountered in disease. The measurement of the proteolytic activity of blood by methods based on the dissolution of a fibrin clot by the plasma or serum has two limitations: first, the end point is often very uncertain, especially when the time of dissolution is prolonged; and second, the fibrin clot may be contaminated with the precursor of the enzyme plasmin, whose activation during the measurement procedure alters the results.[2] It is for these reasons that measurements of the enzyme or enzymes responsible for clinical fibrinolysis should be carried out on a substrate other than fibrinogen or fibrin. However, when casein is used as a substrate, the end products of the reaction are measured either colorimetrically as tyrosine or by optical density at 280 mμ. With these two methods, the blank values are very high when plasma or plasma derivatives are present. In contrast, when the casein is radioactive, the end products are measured by their radioactivity and the blank depends solely upon the substrate without contribution by the other reagents used. Therefore, the method described here is indicated for the measurement of proteolytic enzymes in biologic media and especially for the quantitative study of blood in clinical medicine. The application of this method to the study of a case of acute fibrinolysis observed in a patient is reported elsewhere.[4]

SUMMARY

Casein was tagged with radioactive iodine by a simple method. This was found to be a suitable substrate for the enzyme plasmin. The increase in radioactivity in the trichloroacetic acid filtrate from iodinated casein incubated with the enzyme was taken as a measure of proteolytic activity. The main advantages of the method are, first, that the substrate is completely devoid of plasminogen and, second, that the value of the blank and of the experimental readings depends solely on the substrate and not on the other reagents used.

REFERENCES

1. Tagnon, H. J., Levenson, S. M., Davidson, C. S., and Taylor, F. H. L.: The Occurrence of Fibrinolysis in Shock With Observations on the Prothrombin Time and the Plasma Fibrinogen During Hemorrhagic Shock, Am. J. M. Sc. **211**: 88-96, 1946.
2. Astrup, T.: Fibrinolysis in the Organism, Blood **11**: 781-806, 1956.
3. Lerner, A. B., and Barnum, C. P.: The Ultraviolet Absorption of the Plasma Proteins, Arch. Biochem. **10**: 417-425, 1946.
4. Heuson, J. C., Peers, W., and Tagnon, H. J.: A New Diagnostic and Therapeutic Approach. Fibrinolysis and Hemorrhage, Blood **13**: 874-882, 1958.

RAPID ELECTROPHORETIC SEPARATION OF HEMOGLOBINS

B. Zak, Ph.D., E. M. Eggers, M.D., T. L. Jarkowski, M.D., and
L. A. Williams, B.S.
Detroit, Mich.

A RECENT technique which was employed for the electrophoretic separation of abnormal hemoglobins in dilute agar gel[1] was modified and extended for the purpose of decreasing the time involved in the separation of these mixtures. Whereas previously it had required 4 hours to attain distinguishing qualitative characteristics in the original procedure, the modified method which used a comparatively high potential gradient achieved a similar consideration in 30 minutes.[2] A continuation of the investigation using highly purified agar and an alteration in technique involving a still higher potential gradient and a decreased ionic strength buffer has enabled the time to be shortened further to 15 minutes with no apparent loss in resolution. Thus, one is able to analyze qualitatively a number of bloods on a single electrophoretogram in an almost negligible time interval. As many as 3 plates may be prepared at one time, and each plate can hold 8 samples. Therefore, up to 24 simultaneous determinations can be carried out in this comparatively short period. This makes the procedure quite useful as a screen in mass survey type projects.

MATERIALS AND METHODS

1. Veronal buffer, 0.025 (plate) and 0.05 (boxes), ionic strength, pH 8.6.

2. Agar-buffer gel: Weigh out 175 mg. of agar (purified, Difco Certified) and dissolve it in 100 ml. of buffer by bringing the solution to a boil.

3. Prepared tissue slide: Glue a 2 mm. glass rod onto the edges of a 2 by 3 in. tissue slide using Cenco label varnish No. 11380A.

4. Heathkit power supply and buffer boxes.[2]

5. Wick bridges: Schleicher & Schuell filter paper, No. 900, approximately 5 by 9 cm.

6. Infrared lamp.

Procedure.—Place the prepared tissue slide onto a small level block between the two buffer boxes, and pipette 5 ml. of the hot agar-buffer solution onto the slide. Adjust the liquid in the buffer boxes so that they are at the same level as the liquid on the slide. Connect the buffer boxes to the ends of the slide with the wick bridges which have been previously soaked in buffer and blotted free of the excess material. After a 10-minute waiting period during which the solution has become quite firm, small slivers of Whatman No. 3 mm. paper are soaked in the hemolyzed hemoglobin solutions, blotted on filter paper, and carefully placed onto the agar gel. The Heathkit power source is adjusted to an

From the Departments of Pathology, Wayne State University College of Medicine and Detroit Receiving Hospital, and the Office of the Wayne County Medical Examiner.

Supported in part by a grant-in-aid from the Receiving Hospital Research Corporation and Grant No. A-1660 of the Department of Health, Education and Welfare, National Institutes of Health.

Received for publication Dec. 22, 1958.

applied voltage of 250 volts and electrophoresis allowed to proceed for 15 minutes. The applied voltage is then disconnected, the wick bridges are carefully removed, and the plates dried by means of an infrared lamp.

RESULTS AND DISCUSSION

Several factors were evaluated for this procedure. These included electrophoresis time, buffer strength, agar concentration and purity, electrophoresis path length, and applied potential. It was found that the minimum time for reliable separation of the constituents of a mixture involving A, C, or S hemoglobins was 10 to 15 minutes, and 15 minutes was considered, therefore, to be optimum. It is possible to separate the bands further using a 25- to 30-minute time period when quantitation for heterozygosity is desired. This time of separation was intimately associated with the other variables, especially buffer strength. At low ionic strength, 0.020 to 0.030, the separation of hemoglobins in this agar gel system affords a superior resolution to the conventional 0.05 ionic strength buffer. The agar concentration was increased somewhat over the previous technique using a high purity agar rather than the ordinary Difco Bacto agar. The result was a firm gel which set quickly and handled easily.

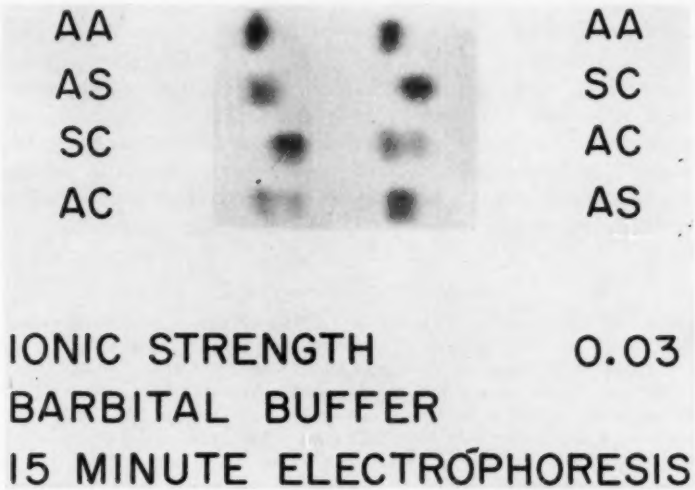


Fig. 1.—Electrophoretogram of several hemoglobins.

The applied potential was maintained at 250 volts and, since the path length had been shortened by 25 per cent over the previous technique,[1] the increased potential gradient allowed a more rapid separation for the two components present. Although other applied potentials could provide reasonable separations, the voltage decided on was considered to be optimum for the described conditions. The actual IR drop across the 7.5 cm. of tissue slide measured at approximately one half the applied potential because of the IR drop loss across the buffer boxes.

Fig. 1 exemplifies the type of distinct separation on a clear background one can obtain using the 15-minute microtechnique with several previously determined samples of normal and abnormal hemoglobins. In comparing the rapid procedure to our routine overnight paper electrophoresis determinations, no discrepancies were encountered. It is apparent that this simple, extremely rapid procedure can achieve useful clinical results. A further study including quantitation of the components of heterozygous samples is presently in progress.

SUMMARY

A simple and rapid microprocedure for the separation and qualitative identification of normal and abnormal hemoglobins in an agar gel medium has been described. The equipment and space required are minimal and the technique agrees well with standard paper electrophoresis, even though much less material and time are required.

REFERENCES

1. Zak, B., and Sun, K. M.: Technic for Separation of Proteins by Means of Agar-Gel Electrophoresis, Am. J. Clin. Path. **29**: 69-79, 1958.
2. Zak, B., and Jarkowski, T. L.: Rapid Electrophoretic Separation of Hemoglobins and Serum Proteins, Clin. chim. acta **3**: 494-496, 1958.

PHOTOELECTRIC ULTRAVIOLET PHOTOMETRY APPLIED TO PROTEIN ELECTROPHORESIS

NEWTON RESSLER, PH.D.
ELOISE, MICH.

ALTHOUGH zonal electrophoresis has proved most useful for routine clinical use, the staining procedures commonly employed are generally recognized to have limitations for quantitative purposes.[1]

A method combining some of the properties of both free solution and zonal electrophoresis utilizes as medium a thin layer or film of buffer solution which is stabilized by such a low concentration of gelling agent that it remains as a fluid.[2] By conducting the electrophoresis over a fused silica or quartz plate, it is possible to follow and quantitate the proteins by means of their absorption of ultraviolet light without drying or staining. The ultraviolet photometry can also be used with other electrophoretic methods with backgrounds transparent to light.

Ultraviolet photometry, conducted by exposing a photographic plate directly under the quartz or silica to monochromatic ultraviolet light coming down through the protein solution has already been described.[3] The measurement of the ultraviolet absorption photoelectrically instead of photographically would have the added advantage of providing direct optical density values without the necessity of calculations. This procedure was investigated, and the results are reported below.

MATERIALS AND METHODS

The electrophoresis was conducted in the same manner that had been employed previously.[2] The gelling agent was dissolved by adding it to the buffer (0.05 mole of tris[hydroxymethyl]aminomethane and 0.05 mole of LiCl per liter, adjusted to pH 8.6 with HCl) and heating to a boil. The solution was layered over a level sheet of fused silica or quartz (0.17 ml. per cm.^{2}) which had been bordered along the edges with plastic or glass strips to prevent the buffer from running out. Contact was made with electrode vessels filled with buffer up to the same level as the film by means of 2 layers of thick filter paper wicks (Whatman No. 17, wet with the buffer). The voltage was applied and a narrow strip of filter paper, about 2 mm. wide, which had been wet with serum was placed by means of forceps in the buffer solution. The buffer composition and film thickness was maintained constant by covering the buffer and sealing the sides with Saran Wrap to prevent evaporation.

Fig. 1 shows a 2 by 10 inch sheet of fused silica, used as electrophoretic cell, after the electrophoresis was finished, and the electrode vessels and filter paper wicks withdrawn. The light source for the photometry was a Bausch & Lomb grating monochrometer, positioned 6 feet above the cell. The wave length setting was 200 mμ.

From the Department of Medicine, Wayne County General Hospital, Eloise, Mich.
This investigation was supported in part by grant A-2726 from the National Institutes of Health, United States Public Health Service.
Received for publication Nov. 26, 1958.

The silica cell was supported from the sides at the electrode ends so as to permit a Beckman DU spectrophotometer photocell to be moved about underneath. The photocell entrance was covered with a diaphragm containing a 2 mm. wide slit opening and was moved over graph paper lined at 2 mm. intervals. By taking readings at every 2 mm. interval, the optical density along the entire migration path was thus measured. The Beckman spectrophotometer was used and balanced in the usual fashion except that the light source was the Bausch & Lomb monochrometer rather than its own lamp housing compartment. A red light, too dim for response of the ultraviolet sensitive photocell, provided sufficient visibility to operate the instrument.

Photographic photometry was conducted according to previous descriptions.[3] Kodak SWR film was used throughout.

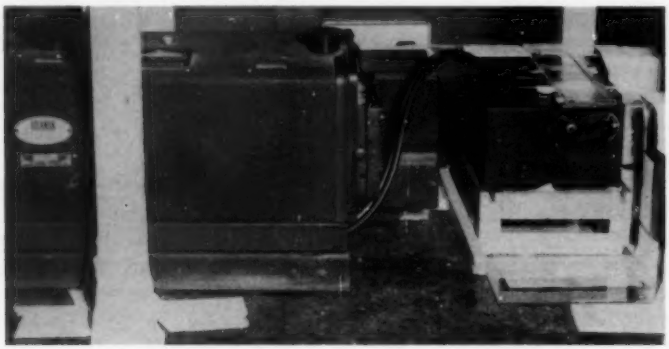


Fig. 1.—Photocell in position to be moved over graph paper so as to measure the optical density along the migration path of the 2 by 10 inch silica cell. The light comes down through the protein solution from a monochrometer positioned on a stand 6 feet above. The photocell entrance is covered with a diaphragm containing a slit opening with a width of 2 mm., the same as the interval of movement.

RESULTS

A solution of 4 human serum proteins, ceruloplasmin,* β_1-metal-combining globulin,* fraction II,† and fraction V,† was prepared by dissolving the dry powder in the tris buffer containing 0.16 per cent agar as gelling agent. A quartz plate was partitioned into a number of water-tight compartments by gluing plastic strips, and 0.17 ml. per cm.^{2} of the various protein solutions was pipetted into each one. The optical density of each of the solutions was then determined by means of the Bausch & Lomb monochrometer and Beckman photocell, and the results are presented in Fig. 2. The proteins appear to have similar extinction coefficients over the concentration range shown; this is in agreement with the results obtained photographically.

Different solutions of the same proteins in amounts of 0.17 ml./cm.^{2} were pipetted onto vinyl sheets, dried by infrared heating lamps, and stained for 25 minutes in a saturated solution of Amido black 10B.[4] The solvent, 50 per cent alcohol in 10 per cent acetic acid, was also used to rinse just sufficiently to clear the background. The optical densities were determined in a densitometer, and are shown in Fig. 3. The variation in staining with different proteins is consistent with the results of previous investigations.[5]

*Obtained from the Michigan State Department of Health.
†Obtained from the American Red Cross.

A sample of human serum was subjected to electrophoresis on a 2 by 10 inch sheet of fused silica. Agar (0.16 per cent) was used as gelling agent. After the proteins were sufficiently separated, the ultraviolet absorption along the migration path was measured photographically. The absorption was then measured photoelectrically, 8 times. The photographic plate and optical density curve are compared to one of the curves obtained photoelectrically in Fig. 4.

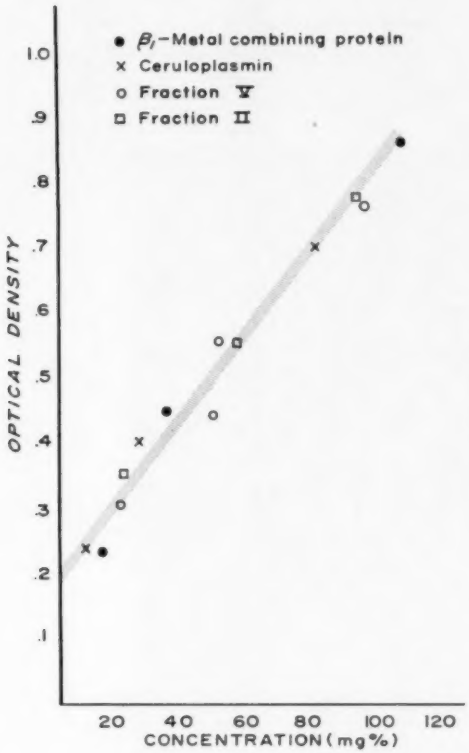


Fig. 2.

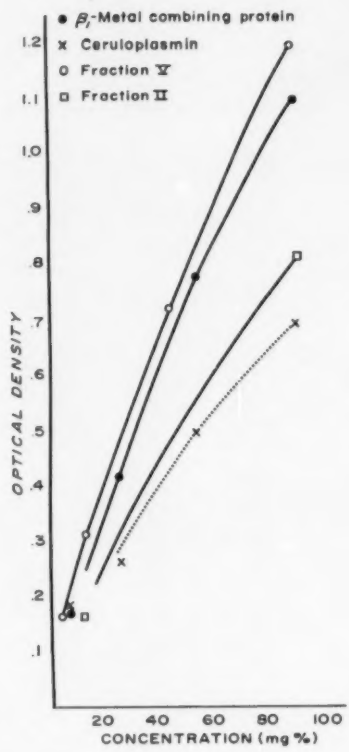


Fig. 3.

Fig. 2.—Optical density, at 200 mμ, of various serum protein solutions measured under electrophoretic conditions.

Fig. 3.—Optical density after staining with Amido black 10B for the same serum proteins as in Fig. 2.

The per cent of the total area under the peak corresponding to each of the serum proteins is given in Table I for the 8 photoelectric determinations, and in Fig. 4 for the photographic determination. The electrophoresis had not been conducted for a sufficient time to separate α_1-globulin from albumin, and the two are measured together.

The separated serum proteins in the buffer solution were next fixed on the silica plate by infrared drying, and the plate was then immersed in Amido

TABLE I. REPRODUCIBILITY OF PHOTOELECTRIC MEASUREMENTS*

	ALBUMIN PLUS α_1-GLOBULIN	α_2-GLOBULIN	β-GLOBULIN	γ-GLOBULIN
	47.3	18.6	14.3	19.8
	51.7	16.0	14.1	18.0
	49.8	17.0	16.0	17.2
	53.8	15.1	14.4	16.7
	50.6	16.1	15.3	17.9
	50.5	16.9	14.7	18.0
	51.0	15.6	14.5	18.9
	52.6	14.2	13.9	19.4
Mean	50.9	16.2	14.6	18.2
Standard deviation	1.82	1.25	0.64	0.99

*Numbers refer to the per cent of the total area under each peak.

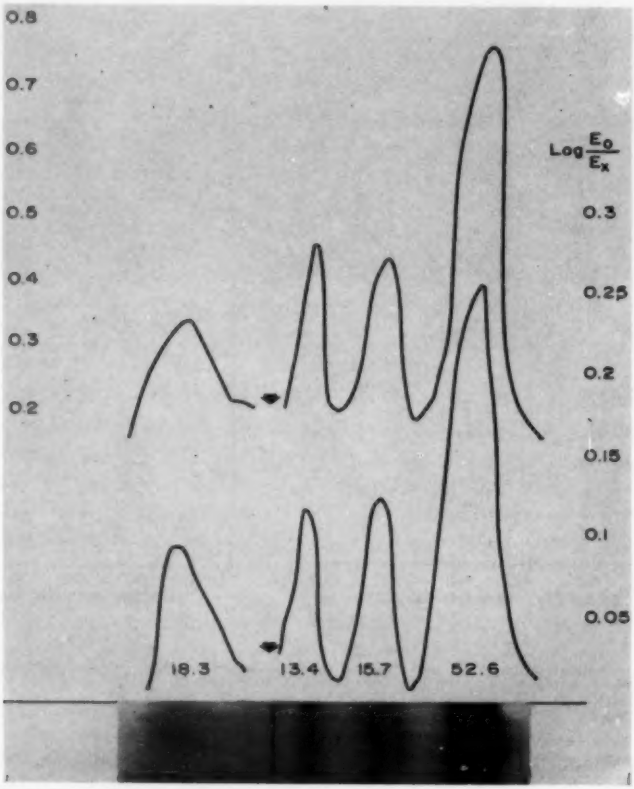


Fig. 4.—Lower optical density curve and electropherogram obtained photographically. Upper optical density curve obtained photoelectrically. Numbers give the per cent of total area under each peak.

black 10B stain. The plate was rinsed in the solvent for about 30 seconds, dried, and the optical density of the Amido black 10B stain along the migration path was measured in a densitometer. The silica plate was then rinsed an

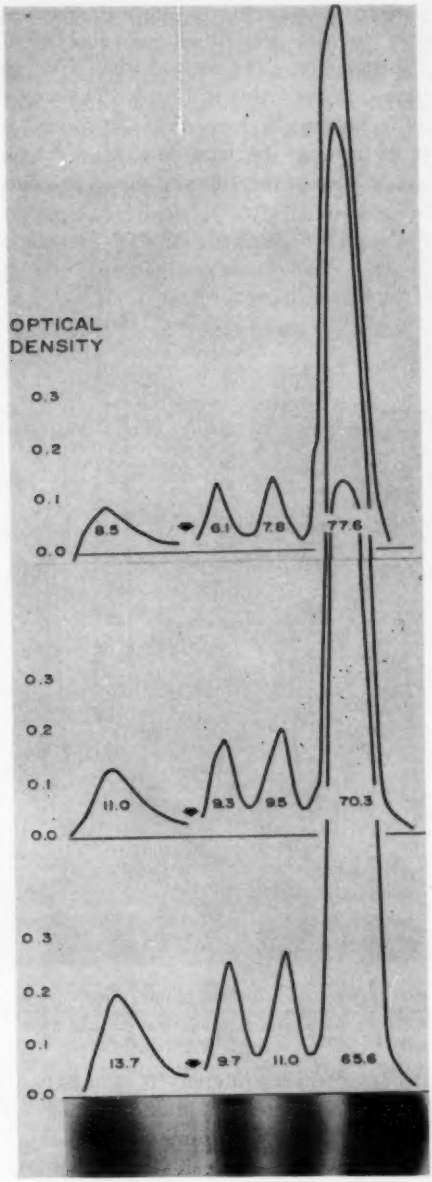


Fig. 5.—The same separation as measured in Fig. 4 after drying and staining. The curves give the Amido black 10B optical density after rinsing for (top to bottom) 20½, 10½, and ½ minutes. Numbers give per cent of total area under each peak.

additional 10 minutes in the solvent; the optical densities were determined a second time and, after the plate was rinsed for a final 10 minutes, the plate was read again in the densitometer. The stained plate after the first rinse and the 3 optical density curves are presented in Fig. 5. The curves show that the stain bound to the protein continues to dissolve in the solvent with further rinsing. This has the effect of raising the base line with corresponding changes in apparent per cent composition of the different serum proteins.

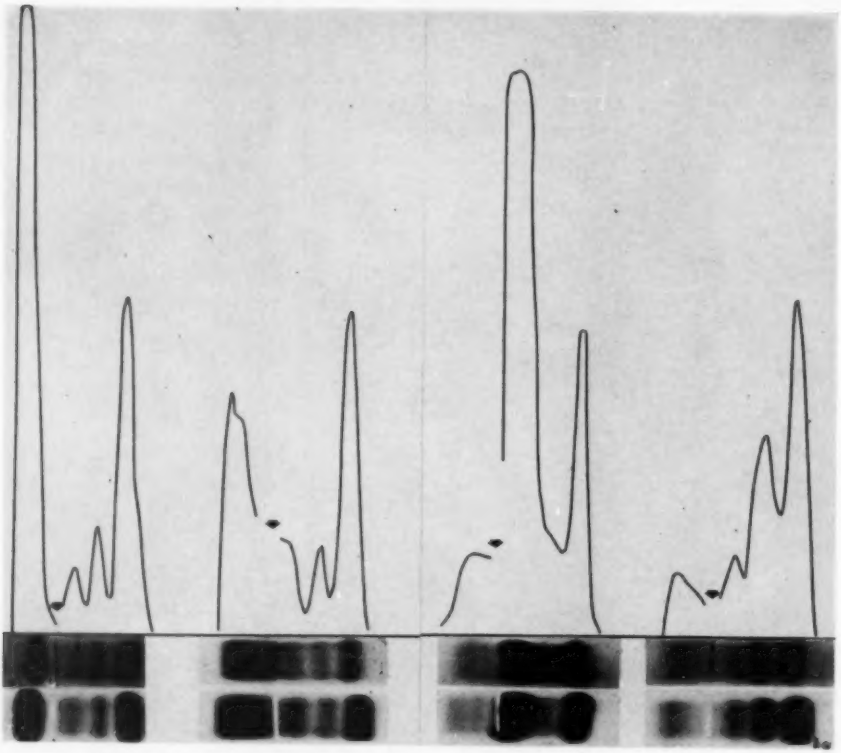


Fig. 6.—Photoelectric curves (top), printed photographic plates (center), and stained electropherograms (bottom) are shown for the same separation for 4 abnormal sera. The potential gradient was 9.4 volts per centimeter, and the running time was 2 hours.

In order to further compare different photometric procedures, an electrophoretic separation was conducted simultaneously on 4 different sera, each exhibiting a marked abnormality in protein composition. A 6-inch quartz square was used as electrophoretic cell; 9.4 volts per centimeter (120 volts, 12.7 cm. distance between wicks) was applied for a period of 2 hours. Photographic exposures were obtained on the 4 sera. Photoelectric measurements were taken next, followed by drying and staining with Amido black 10B. The stained

electropherograms, the printed photographic plates, and the photoelectrically obtained ultraviolet curves are compared for each of the 4 sera in Fig. 6. It is apparent that the same abnormal deviations are indicated by all 3 methods.

Zak and Jarkowski,[6] using a modification of the present procedure, have demonstrated that electrophoretic separations can be obtained in less than an hour, by the use of high potential gradients. This is also illustrated in Fig. 7 which shows printed photographic plates of the same 4 sera used above, after electrophoresis at a potential gradient of 18.9 volts per centimeter for a period of 38 minutes. In this case, 1 per cent potato starch was used as the gelling agent, and the same gross abnormalities were found as with agar. An ice tray was used as a cover during the run, in order to maintain the temperature between 25° and 30° C.

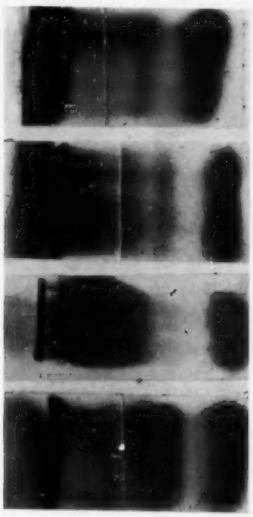


Fig. 7.—Printed photographic plates run with starch gelling agent. The running time was 38 minutes, at 18.9 volts per centimeter.

DISCUSSION

Although most interest concerning the absorption of ultraviolet light by proteins has been with the wave length region of 280 mμ,[7] the far ultraviolet at 200 mμ provides a considerably greater sensitivity. It also has the advantage that a large part of the absorption is due to the peptide linkage,[8] and the various proteins have more similar absorption coefficients. The sensitivity can be improved by a decrease in stray radiation. The protein absorption maximum is at 185 mμ, at which wave length the absorption coefficient is approximately 50 per cent greater than at 200 mμ.[8]

The elimination of the staining procedure avoids or reduces certain variable factors, such as gross differences in staining among different proteins and the problem of rinsing the stain from the background without losing any from

the protein. By performing the photometry directly without drying, the medium retains the optical characteristics of a solution, i.e., a low, homogenous background without reticular or granular structure. It permits the photometry to be conducted without disturbing the electrophoresis, and proteins may be followed during the course of their migration.

The reproducibility obtained above is comparable to that of other methods.[9] An initial blank reading should be made in order to ascertain that the light intensity is sufficiently homogenous throughout the migration path. This would not be necessary with an alternative design in which the light is moved along with the photocell so that the relative position of the two does not change. The reproducibility that can be attained would be the same as that with other spectrophotometric procedures since the basic operations are the same.

With the use of higher potential gradients, an ice tray cover or other cooling device is necessary in order to remove excess heat without evaporation of the film. A temperature range of 25° to 30° C. has been found to be suitable.

The photographic photometry has the advantage of being able to cover a large field in a single exposure. This may be especially important when a large number of samples are being run simultaneously or with two-dimensional electrophoresis. It also provides a visual picture of the protein distribution. The sensitivity of the method can readily be adjusted by the selection of an exposure time suitable for a relatively high or low protein concentration.

The photoelectric method has the advantage of providing optical density values directly without calculations. By using a motor-driven photocell connected to an automatic recorder, the optical density of the proteins along the migration path can be determined for a large number of samples in 1 or 2 hours after applying the voltage. A combination of both photographic and photoelectric photometry may be the most useful arrangement for general purposes.

SUMMARY

A photoelectric method of far ultraviolet photometry has been applied to the electrophoresis of proteins. It is not necessary to disturb the electrophoresis, and proteins can be followed during the course of their migration.

The photometry is direct, without drying or staining, which avoids variables due to these steps and helps make it possible to obtain protein absorption curves along the migration path in relatively short periods of time.

REFERENCES

1. Wolstenholme, G. E., and Millar, E. C. P.: Paper Electrophoresis, Boston, 1956, Little, Brown & Company.
2. Ressler, N., Nelson, N. A., and Oster, H. L.: Fluid Film Method of Electrophoresis, J. Lab. & Clin. Med. **51**: 623, 1958.
3. Ressler, N., and Jacobson, S. D.: Far Ultraviolet Direct Photometry Applied to Protein Electrophoresis, J. Lab. & Clin. Med. **54**: 1959.
4. Smithies, O.: Zone Electrophoresis in Starch Gels, Biochem. J. **61**: 629, 1955.
5. Loeffler, W., and Wunderly, C.: The Staining of Proteins and Lipoids After Electrophoresis on Filter Paper, J. Clin. Path. **6**: 282, 1953.

6. Zak, B., and Jarkowski, T. L.: Rapid Electrophoretic Separation of Hemoglobins and Serum Proteins, Clin. chim. acta **3:** 494, 1958.
7. Beaven, G. H., and Holiday, E. R.: Ultraviolet Absorption Spectra of Proteins and Amino Acids, Advances Protein Chem. **7:** 319, 1952.
8. Goldfarb, R., Saidel, L. J., and Mosovich, E.: Ultraviolet Absorption Spectra of Proteins, J. Biol. Chem. **193:** 397, 1951.
9. Berry, E. R., and Chanutin, A.: Detailed Electrophoretic Analyses of Sera of Healthy Young Men, J. Clin. Invest. **34:** 1513, 1955.

A RAPID METHOD FOR PREPARING WASHED RED CELLS TAGGED WITH CHROMIUM^{51}

CHALOM A. ALBERT, M.D., HAROLD N. ECCLESTON, JR., M.D., AMIR RAFII, M.D., CHARLES H. HUNTER, M.D., EVELYN E. HENLEY, M.D., AND SOLOMON N. ALBERT, M.D.
WASHINGTON, D. C.

MEASUREMENT of the circulating blood volume is a valuable laboratory procedure necessary for the proper management of the medical and surgical patient.[1]

Repeated volume measurements, utilizing red cells tagged with Cr^{51}, seem to be fairly consistent and reproducible. Moreover, the venous hematocrit is a reflection of the volume measured by distribution of the cellular element.[2-4]

Introduction of the plastic coil for measurement of radioactivity in liquid samples, and the preparation of stock O Rh negative tagged cells have contributed in rendering blood volume measurements practical and simple.[5]

In an attempt to further simplify the standard procedure and shorten the time necessary for tagging and washing red cells, the following modifications have been introduced. These are based on a new method for collecting blood and centrifugation.

MATERIALS AND METHODS

Plastic Centrifuge Bag.—A small flat plastic bag (Fig. 1, *a*), with a collecting tube extension and needle adapter was prepared. The capacity of the plastic centrifuge bag is 40 to 45 ml. and, when filled, fits into the standard metal 50 ml. centrifuge tube. The plastic bag is inserted with the collecting tube bent on itself and the bag inlet is introduced into the protector metal tube in an inverted position (Fig. 1, *b*).

Red cells, on centrifugation, separate and are collected at the inlet of the bag. A syringe is attached to the collecting tubing and the washed packed cells are removed (Fig. 1, *c*).

This procedure eliminates the use of long sterile needles and suction apparatus for removing supernatant washing solution from packed cells.

Preparation of Stock O Rh Negative Cells Tagged With Cr^{51}.—One millicurie of sodium Rachromate is added to 1 pint of O Rh negative blood preserved in ACD solution, as obtained from the blood bank. The bottle is shaken continuously while the Cr^{51} is introduced. Frequent agitation for 30 minutes at room temperature is necessary for adequate tagging. The bottle of blood is then filtered into an empty plastic bag (Radibag). The bag is then suspended in the refrigerator at 4 to 6° C. with the inlets of the bag hanging free.

From the Anesthesiology Research Laboratory and Radioisotope Laboratory, District of Columbia General Hospital, Washington, D. C.

Project supported in part by the Atomic Energy Grant AT (30-1) 1820 with the George Washington University.

Received for publication Jan. 19, 1959.

Preparation of Washed Tagged Cells.—Ten to 15 milliliters of packed cells are transferred into a centrifuge bag from the stock preparation. The bag is filled with chilled normal saline solution and centrifuged in an inverted position. At the completion of spinning, the centrifuge bag is removed and suspended. The washed packed cells are withdrawn in a 20 ml. syringe. A sufficient quantity of normal saline is added in order to resuspend the washed tagged cells to approximately 18 ml.

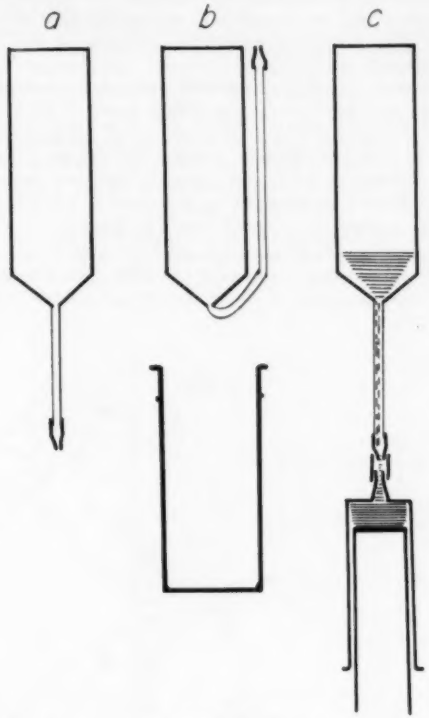


Fig. 1.—*a*, Centrifuge bag. *b*, Centrifuge bag with collect tube bent to be inserted in metal centrifuge container. *c*, Removal of packed cells from centrifuge bag.

After thorough shaking, a portion of the red cell suspension is injected into the plastic coil for counting—this represents the count of standard—and an accurately measured 10 ml. volume of the remaining portion is injected to the patient.

A multidose washed tagged red cell suspension can be prepared in a similar manner. The washed cells are mixed in a 250 ml. plastic bag, and normal saline is added in order to prepare a red cell suspension with a radioactivity count of 150,000 to 200,000 counts per minute. A sample of the red cell preparation is used to obtain the standard count. Doses of 10 ml. are used for each volume determination. The count of the standard varies very little over a 12-hour period, and repeated counting of standard is not necessary.

Rapid Method for Tagging Blood Removed From Recipient.—Tagging and washing of patients' cells is made in one single step. Twenty milliliters of blood is removed from the patient directly into a centrifuge bag, containing 4 ml. of ACD solution and 50

μc of sodium Rachromate. The bag is shaken while blood is withdrawn. The centrifuge bag is filled with normal saline and centrifuged immediately. A suspension of tagged cells is prepared as mentioned in the previous section.

The amount of free chromium trapped between the cells varies between 0.75 and 1.85 per cent with a mean of 1.23 per cent. Correction of the count obtained as standard by 1 per cent would correct this error.

Duration and Speed of Centrifugation.—Clinical centrifuges with a standard type of 90-degree head (radius 14.1 cm.) revolve at maximum speed of 3,100 r.p.m. (1,510 g). The time required in order to obtain adequate separation of the cellular elements is 20 minutes of spinning time.

Red cells subjected to varying speeds and duration of centrifugation were studied as to fragility, red cell count, and 12-hour survival in vivo.

No adverse effect on red cells was noted at a rotation speed of 10,500 r.p.m. (14,000 g) for 5 and 10 minutes. Beyond 10,500 r.p.m. (14,000 g) red cells are destroyed rapidly. The minimum rotation speed and duration necessary to obtain adequate separation of cells varies between 4,000 to 4,500 r.p.m. (2,400 to 2,500 g) for 5 to 7 minutes.

With the clinical centrifuge a rotation speed of 4,500 r.p.m. and 2,400 g can be obtained by replacing the standard 90-degree angle head with a 45-degree angle head having a 10.8 cm. radius (International Equipment Company, Cat. No. 804).

Total spinning time necessary in order to obtain satisfactory red cell separation is 7 to 8 minutes (Fig. 2).

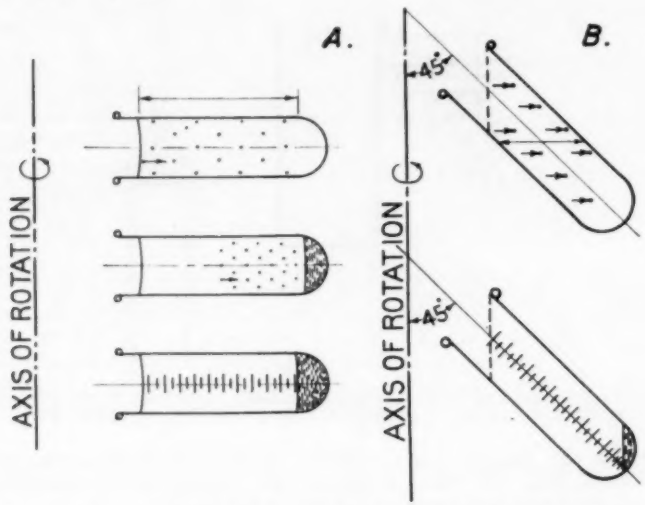


Fig. 2.—*A*, Axis rotation of 90 degrees. Standard head radius of 14.1 cm. Maximum speed of 3,100 r.p.m. (1,510 g). *B*. Axis rotation of 45 degrees. Head No. 804. Radius of 10.8 cm. Maximum speed of 4,500 r.p.m. (2,400 g). (Courtesy International Equipment Company.)

SUMMARY

The plastic centrifuge bag for washing tagged cells simplified and eliminated sources of contamination in the manipulation of cells during tagging and washing procedures. An angle centrifuge head adapted to the regular clinical centrifuge reduces the time necessary to obtain adequate separation of tagged cells by one half.

Acknowledgment is made to The Abbott Laboratories, North Chicago, Ill., for assistance in the development of the Radibag and RadiCentrifuge Bag; and to the International Equipment Company for their cooperation and permission to reproduce Fig. 2.

REFERENCES

1. Albert, C. A., Thergaonkar, R. D., Henley, E. E., Bain, M., Rafii, A., and Albert, S. N.: The Value of Blood Volume Determinations in Surgical Procedures, Surg. Gynec. & Obst. **107**: 685-689, 1958.
2. Albert, S. N., Spencer, W. A., Albert, C. A., Shibuya, J., and Henley, E. E.: Part I. Blood Volume Determinations With Radioactive Isotopes and Observations on Blood Volume Fluctuations. Part II. Index of Cardiac Clearance; U. S. Atomic Energy Commission, Technical Information Service, Dept. of Commerce (AECU-3614), Washington 25, D. C., March, 1958.
3. Thistlethwaite, J. R., Spencer, W. A., and Albert, S. N.: Blood Volume Fluctuations Determined by Radioisotopes of Chromium and Radioactive Iodinated Serum Albumin, Surg. Gynec. & Obst. **105**: 34-38, 1957.
4. Albert, S. N., Spencer, W. A., Shibuya, J., Coakley, C. S., and Thistlethwaite, J. R.: Observations on Fluctuations in Blood Volume as Determined With Radioactive Isotopes, Anesth. et Analg. **36**: 54-61, 1957.
5. Albert, S. N., Spencer, W. A., Finkelstein, M., Shibuya, J., Alpert, S., and Coakley, C. S.: A Plastic Coil Simplifying Radioactive Liquid Phase Counting, J. LAB. & CLIN. MED. **48**: 471-475, 1956.

USE OF AN ARTIFICIAL STANDARD FOR HEMOGLOBIN DETERMINATION

NEWTON RESSLER, PH.D., NORMAN A. NELSON, M.D., AND
IRENE M. SMITH, B.S.
ELOISE, MICH.

THE Division of Medical Sciences of the National Academy of Sciences–National Research Council, at the request of the Hematology Study Section of the National Institute of Health, organized a panel in 1954 to investigate the standardization of hemoglobin determinations. The recommendations of the Panel were recently reported by Cannan,[1] and it was urged that a standard hemoglobin method be widely adopted. The method considered most suitable was the photometric measurement of hemoglobin as cyanmethemoglobin.[2]

It was also proposed that cyanmethemoglobin solutions, prepared and stored under sterile conditions,[3] be used as standards. Cyanmethemoglobin was recommended tentatively, however, since dilute protein solutions are known to be relatively unstable and subject to bacterial contamination.[4] The development of a more stable standard was urged by the Panel.

While a number of stable, inorganic solutions absorb light at 540 mμ, an artificial solution with the same spectral characteristics as cyanmethemoglobin would be difficult to compose. The cyanmethemoglobin absorption band, however, is broad and nearly flat from 537 to 545 mμ. By isolating this spectral region with a spectrophotometer, it is possible to use as an artificial standard any solution which has a flat, and thus parallel, absorbancy curve over these wave lengths. The absorbancy outside the range of 537 to 545 mμ would be eliminated, and the curves need not match over any other region. A solution of $CuSO_4$ has been proposed by Drabkin as a standard.[5] Its absorbancy increases with increasing wave length at 540 mμ, however, and does not parallel the cyanmethemoglobin curve at its maximum absorbancy.

A solution containing a mixture of $NiCl_2$ and $CoCl_2$, stable at room temperature and unfavorable for bacterial growth, has been prepared with spectral characteristics corresponding to those of the cyanmethemoglobin absorbancy peak. The use of this solution as an artificial standard for the determination of hemoglobin concentration has been investigated and the results are reported below.

MATERIAL AND METHODS

Preparation of Inorganic Solutions.—These were prepared by dissolving appropriate quantities of reagent grade $CoCl_2 \cdot 6H_2O$ and $NiCl_2 \cdot 6H_2O$ in 10 per cent acetic acid.

From the Department of Medicine, Wayne County General Hospital, Eloise, Mich.
Received for publication Jan. 6, 1959.

Hemoglobin Standard.—Cyanmethemoglobin standard was obtained from the Ortho Pharmaceutical Company and was used 8 months in advance of the expiration date.

Photometry.—Hemoglobin was measured as cyanmethemoglobin by the method of Drabkin and Austin.[2] All readings were made on a Beckman DU spectrophotometer, except when indicated otherwise.

Preparation of Artificial Standard.—Dissolve 43.3 Gm. of $NiCl_2 \cdot 6H_2O$ and 1.03 Gm. of $CoCl_2 \cdot 6H_2O$ in 100 ml. of 10 per cent acetic acid. The solution can be stored indefinitely in an absorption cell by sealing to prevent evaporation. Allowing an air space permits mixing by inversion, in case any bubbles should form. Filter.

Procedure for Calibration.—Determine the optical density of the artificial standard solution at 542 mμ, in any spectrophotometer having a half intensity band width of 5 mμ or less. Set the optical density of the artificial standard equivalent to 60.1 mg. hemoglobin per 100 ml., and calculate the concentration of an unknown cyanmethemoglobin solution by application of Beer's law.

Spectrophotometers having a half intensity band width greater than 5 mμ will be discussed later.

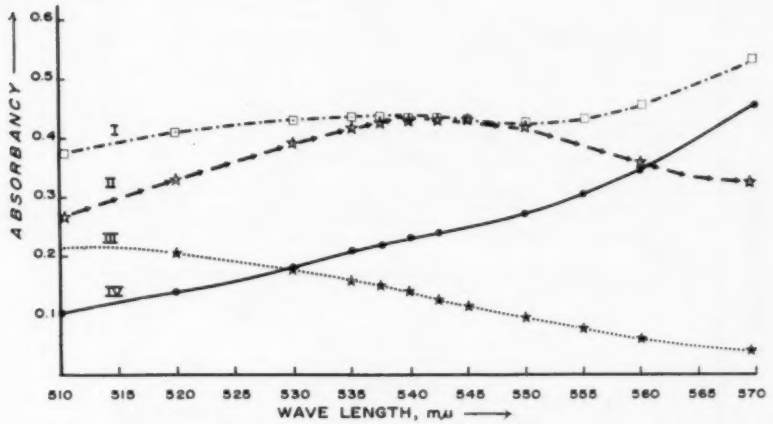


Fig. 1.—Absorbancy versus wave length of solutions containing (I) both 1.03 per cent $CoCl_2 \cdot 6H_2O$ and 43.3 per cent $NiCl_2 \cdot 6H_2O$, (II) 60.1 mg. per cent cyanmethemoglobin, (III) 1.03 per cent $CoCl_2 \cdot 6H_2O$, and (IV) 43.3 per cent $NiCl_2 \cdot 6H_2O$.

RESULTS

Fig. 1 shows the absorbancy of a solution containing 1.03 Gm. per cent $CoCl_2 \cdot 6H_2O$, a solution containing 43.3 per cent $NiCl_2 \cdot 6H_2O$, and a combined solution of both (the solvent in all cases being 10 per cent acetic acid). As the wave length increases from 510 to 570 mμ, the absorbancy of the $CoCl_2 \cdot 6H_2O$ decreases, while the absorbancy of the $NiCl_2 \cdot 6H_2O$ increases. The absorbancy of the combined solution is nearly flat from 535 to 555 mμ and runs closely parallel to the cyanmethemoglobin absorption curve from 540 to 545 mμ.

The absorbancy versus concentration curves for $CoCl_2 \cdot 6H_2O$ and for $NiCl_2 \cdot 6H_2O$ are presented in Fig. 2.

The absorbancy of the solution containing both $NiCl_2 \cdot 6H_2O$ and $CoCl_2 \cdot 6H_2O$ (shown in Fig. 1) is greater than the sum of the absorbancies of the separate solutions. The absorbancy curve of the solution containing both salts

is compared to the sum of the absorbancies of the 2 separate solutions (Fig. 1) in Fig. 3, *A*. The difference appears to be due to complex formation between Co^{++} and Cl^-, since the ability of cobaltous ions to form halide complexes is well known, and the presence of $NiCl_2$ provides a large concentration of Cl^- ions. This is confirmed by the increase in absorbancy of the $CoCl_2$ solution upon the addition of NaCl, Fig. 3, *B*. The addition of NaCl to a solution of $NiCl_2$ did not increase the absorbancy at 540 mμ. The difference in absorbancy between the mixed solution and the sum of the separate solutions increases with increasing concentration of Co^{++} ions (Fig. 4, *A*) and Cl^- ions (Fig. 4, *B*). Since the absorbancy is linear with concentration in both cases, the usefulness of the mixed solution as a standard is not affected.

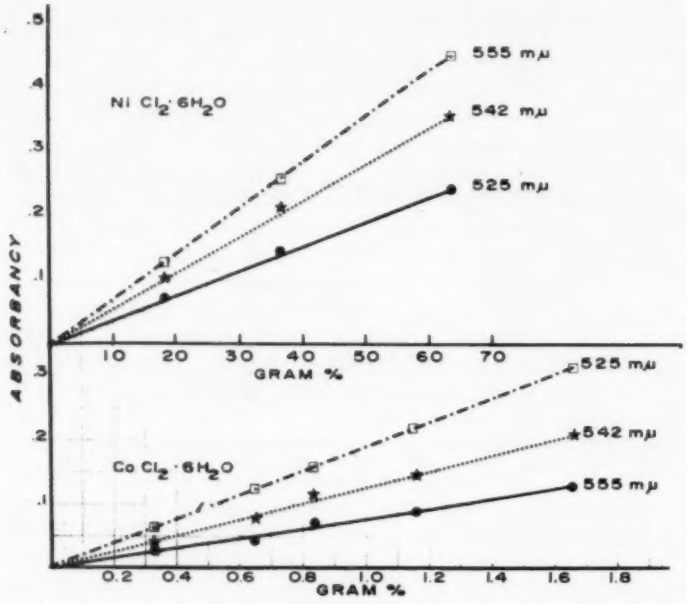


Fig. 2.—Absorbancy versus concentration for $NiCl_2 \cdot 6H_2O$ and $CoCl_2 \cdot 6H_2O$ at indicated wave lengths.

Cobaltous ions produce intensely blue colored solutions in some instances. The blue form is favored by the addition of HCl and by high temperature.[6] In order to determine if an unstable equilibrium might be involved between the blue and pink colors, additions of HCl were made to a portion of the standard solution. No color change was observed until after the solution had been diluted several hundred per cent. Another portion of the standard solution was heated for 20 minutes in a boiling water bath, and its absorbancy determined at 540 mμ while still hot. The readings had increased a few per cent, but when the solution was cooled to room temperature, the original readings were again obtained, indicating the stability of the solution. In a further

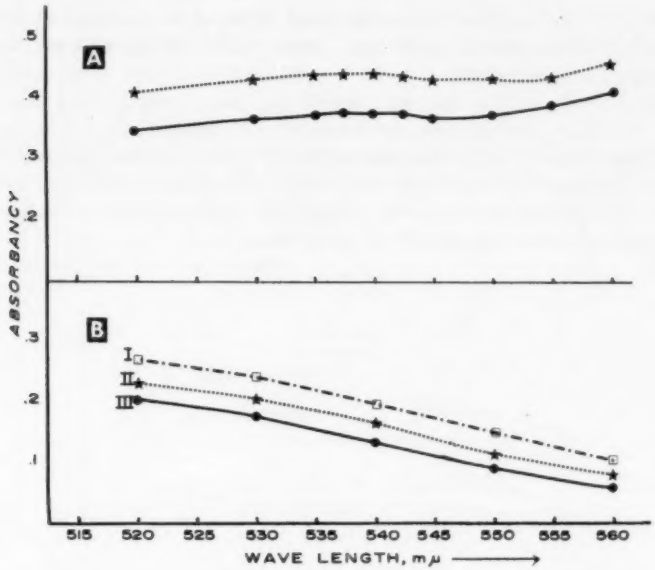


Fig. 3.—*A*, Upper curve is absorbancy of combined nickel and cobalt solution (curve I in Fig. 1), while lower curve is sum of absorbancies of separate solutions (curves III and IV of Fig. 1). *B*, Absorbancy of a 1.03 per cent solution of $CoCl_2 \cdot 6H_2O$ containing (I) 23.75 per cent NaCl, (II) 11.88 per cent NaCl, and (III) 0 per cent NaCl.

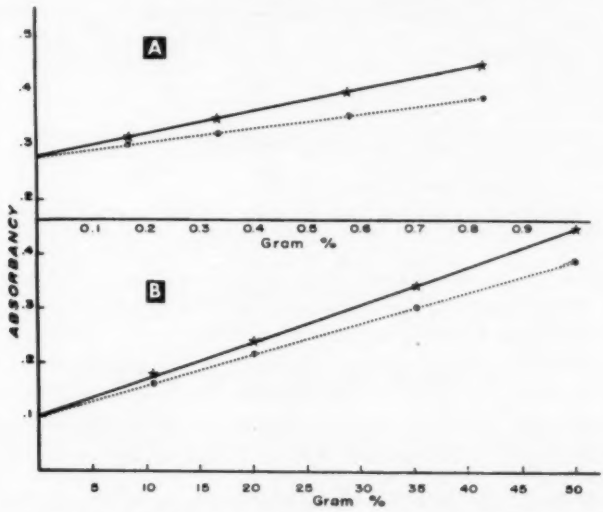


Fig. 4.—Absorbancy at 542 mμ of (A) a solution containing 50.33 per cent $NiCl_2 \cdot 6H_2O$ with variations in $CoCl_2 \cdot 6H_2O$ concentration, and (B) a solution containing 0.83 per cent $CoCl_2 \cdot 6H_2O$ with variations in $NiCl_2 \cdot 6H_2O$ concentration. In both cases, the solid line is the combined solution, and the dotted line is the sum of absorbancies of the separate solutions.

test of stability, the solution was maintained in a hot water bath at approximately 62° C. for a period of 30 days, after which the absorbancy was unchanged from the original value.

Another sample of the solution, which had been prepared by the usual, nonsterile laboratory techniques and stored for 2 weeks in a half-filled container, was centrifuged for 30 minutes at 10,000 r.p.m. and the sediment examined for the presence of bacteria, with negative results.* Culture attempts also failed to indicate any bacteria. The high concentration of nickel ions appears unfavorable for the growth of most bacteria.

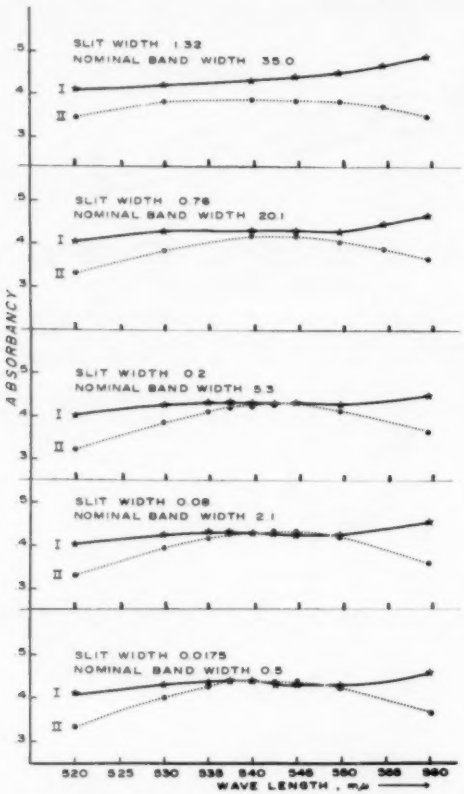


Fig. 5.—Absorbancy of (I) artificial standard and (II) 60.1 mg. per cent cyanmethemoglobin determined with a Beckman DU spectrophotometer at various slit width settings, as indicated, in millimeters. Corresponding band widths at 540 mμ are also shown.

The absorbancies of the artificial standard and of a 60.1 mg. per 100 ml. solution of cyanmethemoglobin were determined in a Beckman DU spectrophotometer at slit widths corresponding to half intensity band widths ranging

*Bacterial examinations were performed by Eugene M. Britt, Ph.D., Department of Pathology, Wayne County General Hospital, Eloise, Mich.

from 0.5 to 35.0 mμ, and the results are presented in Fig. 5. The measurements were also made with Bausch & Lomb Spectronic 20 and Coleman Junior spectrophotometers as shown in Fig. 6.

DISCUSSION

Instrument errors causing alterations in absorbancy values tend to be canceled out by a comparison of 2 absorbancies, the artificial standard and unknown, in the same manner that they would be minimized with a hemoglobin standard. Stray light errors are usually negligible at 540 mμ, due to high photocell sensitivity at this wave length.

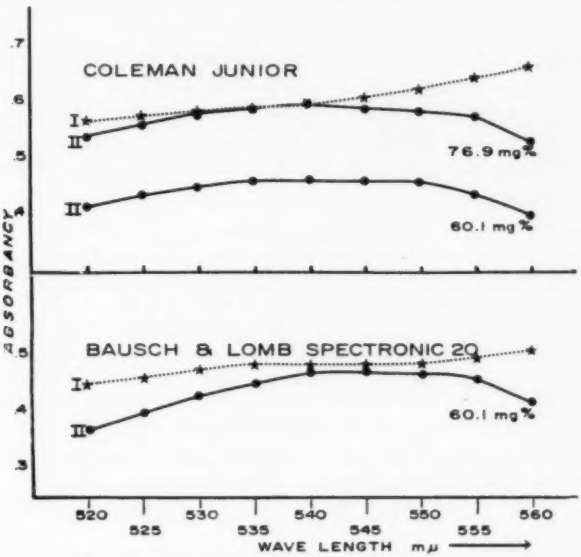


Fig. 6.—Absorbancy of (I) artificial standard and (II) cyanmethemoglobin solutions determined with Coleman Junior and Bausch & Lomb Spectronic 20 spectrophotometers.

By isolating with a spectrophotometer a narrow band of the spectrum, the standard and cyanmethemoglobin need only parallel each other through the width of this band. Each absorbancy reading obtained represents the average absorbancy throughout the width of the isolated band.[7] Since the absorbancy of the cyanmethemoglobin decreases in either direction from the 537 to 545 mμ peak faster than does the standard, it is apparent that the ratio of the absorbancies of the standard to cyanmethemoglobin must increase when the band width becomes greater than the 8 mμ wide peak. This is seen in Fig. 5 to be the case with the 20.1 and 35.0 band widths employed with the Beckman spectrophotometer.

Commercial spectrophotometers used for clinical purposes may have band widths greater than 8 mμ, and the value of the standard will then correspond

to a cyanmethemoglobin concentration greater than 60.1 mg. per 100 ml. The standard corresponded to a cyanmethemoglobin concentration of 62.0 mg. per 100 ml. on the Bausch & Lomb Spectronic 20, compared to the value of 62.2 obtained with the Beckman spectrophotometer, used with a nominal band width of 20.1 mμ. With the Coleman Junior spectrophotometer, the standard corresponded to a cyanmethemoglobin concentration of 76.9 mg. per 100 ml. The results of Fig. 6 indicate that 545 mμ would be the most suitable wave length to use with the Spectronic 20, while 537 appears more appropriate for the Coleman Junior spectrophotometer. Since the compared curves are nearly parallel at the wave lengths used, small errors in wave length calibration will not affect the results. The parallelism can readily be verified by taking readings in either direction.

Some difficulty may be involved in weighing out the $NiCl_2 \cdot 6H_2O$, due to the gain or loss of water. The absorption coefficient of $NiCl_2$ is low at 540 mμ, however, and a weighing error of 500 mg. per cent introduces an optical density error of less than 0.004. The salt can be dehydrated by heating and weighed in the anhydrous state if desired. The $CoCl_2$ used did not change weight during the weighing.

SUMMARY

An artificial standard has been described for use in conjunction with the cyanmethemoglobin method of hemoglobin determination. The standard can be readily and inexpensively prepared in any laboratory. It has an absorbancy curve closely parallel to the peak of the absorbancy curve of cyanmethemoglobin, and is apparently stable and not subject to contamination by most bacteria.

REFERENCES

1. Cannan, R. K.: Proposal for a Certified Standard for Use in Hemoglobinometry, J. Lab. & Clin. Med. 52: 471, 1958.
2. Drabkin, D. L., and Austin, J. H.: Spectrophotometric Studies; Preparations From Washed Blood Cells; Nitric Oxide Hemoglobin and Sulfhemoglobin, J. Biol. Chem. 112: 51, 1935.
3. Crosby, W. H., and Houchin, D. N.: Preparing Standard Solutions of Cyanmethemoglobin, WRAIR-77-57, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C., 1957.
4. Haurowitz, F.: Chemistry and Biology of Proteins, New York, 1950, Academic Press, Inc.
5. Drabkin, D. L.: The Standardization of Hemoglobin Measurement, Am. J. Med. Sc. 215: 110, 1948.
6. Sidgwick, N. V.: The Chemical Elements and Their Compounds, London, 1950, Oxford University Press.
7. Willard, H. H., Merritt, L. L., and Dean, J. A.: Instrumental Methods of Analysis, New York, 1951, D. Van Nostrand Company.

THE ESTIMATION OF TOTAL BODY WATER USING A COLORIMETRIC METHOD FOR THE DETERMINATION OF ANTIPYRINE (1, 5-DIMETHYL-2-PHENYL-3-PYRAZOLONE) IN PLASMA

D. MENDELSOHN, M.B.B.CH., B.SC. (HONS.), AND N. W. LEVIN, M.B.B.CH.*
JOHANNESBURG, SOUTH AFRICA

ALTHOUGH deuterium oxide and tritium oxide are theoretically to be preferred for the determination of total body water in man by the dilution principle, some objections have been raised against the use of these substances. First, it has been recognized that the labeled isotope readily exchanges with labile hydrogen of the tissues resulting in falsely high values for body water.[1-3] Second, the increased mass of deuterium and tritium relative to protium makes it probable that the distribution kinetics of deuterium and tritium oxides are different from ordinary water.[4] Finally, the fact that elaborate apparatus is required precludes determination of deuterium and tritium in many routine laboratories. Of the numerous organic substances which have been used to measure total body water, antipyrine† has been shown to have a volume of distribution conforming well with that of deuterium oxide in normal subjects and in subjects with abnormal accumulations of fluid.[5, 6] Also, results obtained by comparing the antipyrine space with desiccation measurements of total body water in animals agreed very closely.[7] Thus, antipyrine has been extensively employed in clinical investigation to measure total body water. It must be mentioned, however, that when given in repeated doses there is a danger of agranulocytosis.[8]

In the original method for the determination of antipyrine in biologic fluids as described by Brodie and associates,[9] antipyrine is nitrosated in an acid solution to form 4-nitrosoantipyrine, the optical density being measured in a spectrophotometer at 350 mμ before and 20 minutes after the addition of sodium nitrite. Ikkos and co-workers[10] criticized this method stating that the time necessary for the completion of the reaction between antipyrine and sodium nitrite was different from day to day and that readings had to be taken at 10-minute intervals until maximal extinctions were reached. Other investigators[11, 12] have experienced similar difficulties with this technique. Davidsson and MacIntyre[13] reported that it was possible to obtain consistent results if the

From the Departments of Pathology and Microbiology (Division of Chemical Pathology) and Medicine, University of the Witwatersrand, and the Johannesburg General Hospital.
Received for publication Dec. 11, 1958.

*Senior Research Bursar, Cardio-Pulmonary Unit of the Council for Scientific and Industrial Research.

†We wish to express our thanks to Eli Lilly & Company for generously supplying the antipyrine.

temperature were kept constant at 22° C. during the process of nitrosation (25 minutes), but Zak[12] claimed that it was more important to control the nitrite concentration of the reaction mixture, as similar results were obtained at temperatures varying from 22° to 30° C. In view of the above objections to the spectrophotometric procedure and also because many routine laboratories are not equipped with a spectrophotometer it was thought worth while to explore the possibilities of colorimetry.

Delaville and associates[14] determined antipyrine in plasma by a colorimetric procedure using *p*-dimethylaminobenzaldehyde. A disadvantage of this method is the fact that about 3 hours are required for maximal color development. The colorimetric procedure to be described is technically simple, reasonably rapid, and has also the added merit of an increase in sensitivity over the preceding methods.

MATERIALS AND METHODS

Principle of the Method.—The present method has been adapted for colorimetric analysis from a spot test by Feigl and associates.[15] An aliquot of a protein-free filtrate containing antipyrine is nitrosated in glacial acetic acid with sodium nitrite and the resulting nitroso compound condensed with 1-naphthylamine to form an azo dye.

Reagents.—

1. Cadmium reagent: 104 Gm. cadmium sulfate ($3CdSO_4 \cdot 8H_2O$) and 508 ml. 1.0 N H_2SO_4 are made up to 1 liter with water.

2. Sodium hydroxide, 1.1 N.

3. Glacial acetic acid, analytical reagent grade.

4. Sodium nitrite, 15 per cent.

5. Ammonium sulfamate, 75 per cent.

Solutions 4 and 5 are stored in a refrigerator and should be prepared freshly each week.

6. 1-Naphthylamine.* A 10 per cent solution of 1-naphthylamine in glacial acetic acid is made up immediately before use.

7. Stock standard antipyrine (200 μg/ml.). Twenty milligrams antipyrine is dissolved in 100 ml. water. A working standard containing 20 μg/ml. antipyrine is prepared by diluting the stock solution 10 times.

Procedure.—One milliliter of cadmium reagent is added to 2 ml. plasma in a centrifuge tube with mixing. One milliliter 1.1 N sodium hydroxide is then added and the contents well stirred with a glass rod. The tube is centrifuged at 3,000 r.p.m. for 5 minutes and then at approximately 1,500 r.p.m. for 3 to 4 minutes. This procedure produces a perfectly clear supernatant liquid. The following glass-stoppered tubes are now prepared: blank, 1.5 ml. water; standard, 1 ml. working standard + 0.5 ml. water; test, 1.5 ml. protein-free filtrate. Five milliliters glacial acetic acid followed by 0.25 ml. sodium nitrite is added to each tube. The contents are mixed and allowed to stand for 20 minutes, then 0.25 ml. ammonium sulfamate is added and each tube is shaken vigorously until no more gas is evolved. This usually requires 3 to 5 minutes. One milliliter freshly prepared 1-naphthylamine is now added with mixing, and the tubes are placed in a water bath maintained at approximately 50° C. The temperature of the water bath is not critical. After 5 minutes the tubes are removed, cooled in a beaker of cold water, and then allowed to stand at room

*Obtained from Merck & Company, Darmstadt, Germany. All the commercially available brands of 1-naphthylamine (α-naphthylamine) tested were found to be slightly colored. In an attempt to obtain a pure white product, we tried recrystallization from alcohol and water, but without success. Since 1-naphthylamine rapidly becomes oxidized when exposed to the atmosphere it is not possible to obtain a colorless solution in glacial acetic acid without resorting to impractical measures to prevent discoloration. 1-Naphthylamine hydrochloride is no better in this respect. The Merck "pure white" 1-naphthylamine gave the lowest blank values.

temperature for a further 20 minutes. The colors are read in an Evelyn photoelectric colorimeter setting the blank at 100 per cent transmission using a 580 mμ filter and the 6 ml. aperture.

Calculation.— $\frac{T}{S} \times \frac{20}{0.75}$ = micrograms of antipyrine per milliliter of plasma, where T and S are the optical densities of the test and standard (20 μg antipyrine), respectively.

RESULTS

Calibration Curve.—The relationship between optical density and antipyrine in micrograms as determined by the above method is shown in Fig. 1, A. The curve obeys the Beer-Lambert law between the concentrations of 0 to 50 μg antipyrine. This range adequately covers the amount of antipyrine likely to be found in plasma when this substance is being used to measure total body water. The slope of the curve is sufficiently steep to enable one to obtain accurate results with small differences in plasma concentrations of antipyrine.

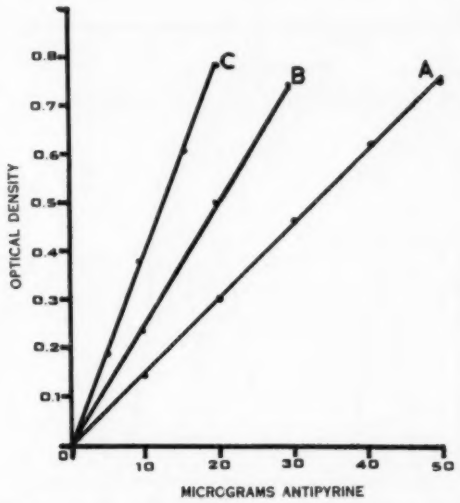


Fig. 1.—Calibration curves using the procedure described. A = 0 to 50 μg antipyrine per 1.5 ml. 25 minutes after the addition of 10 per cent 1-naphthylamine in glacial acetic acid. B = 0 to 30 μg antipyrine per milliliter 1 hour after the addition of 0.5 per cent aqueous solution of N-naphthylethylenediamine dihydrochloride. C = 0 to 20 μg antipyrine per milliliter 6 hours after the addition of 0.5 per cent aqueous solution of N-naphthylethylenediamine dihydrochloride.

Conditions Affecting Color Development.—

Concentration of sodium nitrite: Using the procedure described above with 20 μg/1.5 ml. antipyrine, it can be seen that there is a progressive increase in the intensity of color with increasing concentrations of sodium nitrite (0 to 15 per cent) (Fig. 2). Higher concentrations of sodium nitrite caused no further enhancement of the color. Fig. 3, B, illustrates the absorption spectrum of the diazo compound formed between antipyrine and 1-naphthylamine when 15 per cent sodium nitrite is used. The dye is purple in color with E_{max}

580 mμ. When lower concentrations of sodium nitrite are employed (e.g., 5 per cent), a less intense diazo compound is formed with E_{max} 540 mμ (Fig. 3, A).*

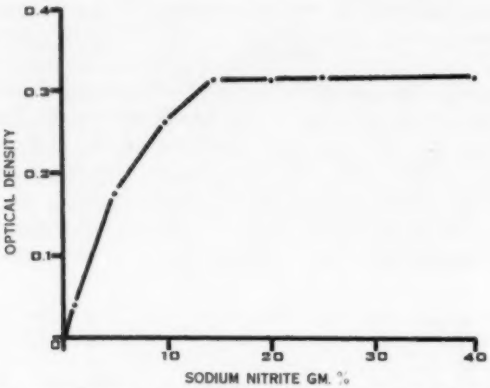


Fig. 2.—Effect of concentration of sodium nitrite on intensity of color using 10 per cent 1-naphthylamine (20 μg antipyrine per 1.5 ml.).

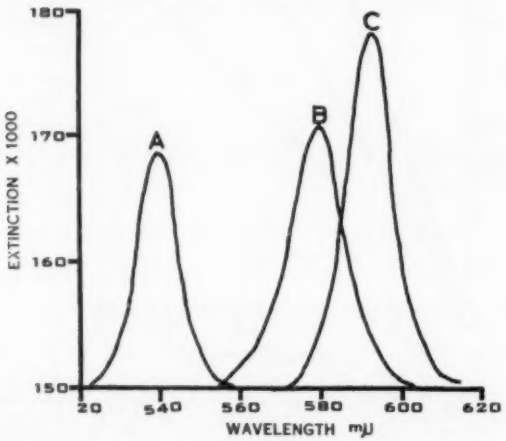


Fig. 3.—Absorption spectra of diazo compounds formed. A = 5 per cent sodium nitrite and 10 per cent 1-naphthylamine in glacial acetic acid. B = 15 per cent sodium nitrite and 10 per cent 1-naphthylamine in glacial acetic acid. C = 15 per cent sodium nitrite and 0.5 per cent aqueous solution of N-naphthylethylenediamine dihydrochloride.

Period of nitrosation: As can be seen from Fig. 4, the intensity of color is related to the period of nitrosation. It was found that the length of time of nitrosation required to give maximum color development varied from day to day between 10 and 20 minutes. However, in no case was it necessary to employ a period of nitrosation longer than 20 minutes.

*No conclusions are drawn as to the nature of the two dissimilar diazo compounds so formed.

Concentration of acetic acid: For the development of maximum color the concentration of acetic acid should be 75 per cent (Fig. 5). There is no further increase in optical density at higher concentrations of acetic acid.

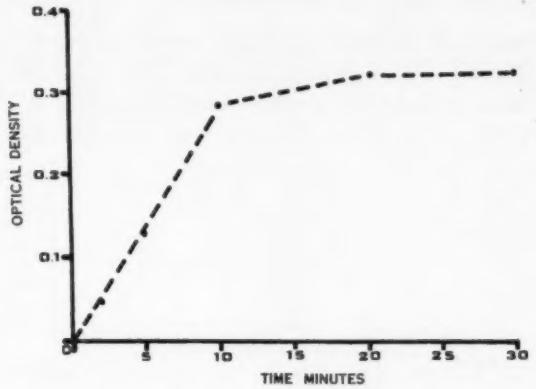


Fig. 4.—Effect of length of time of nitrosation on intensity of color using 10 per cent 1-naphthylamine (20 μg antipyrine per 1.5 ml.).

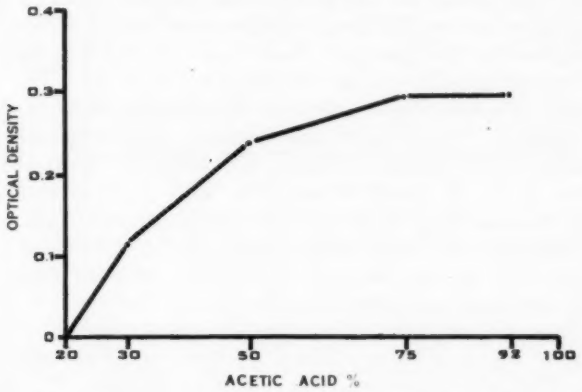


Fig. 5.—Effect of concentration of acetic acid on intensity of color using 10 per cent 1-naphthylamine (20 μg antipyrine per 1.5 ml.).

Intensity and Stability of the Color.—Maximum color intensity is developed 25 minutes after the addition of 1-naphthylamine (Fig. 6, A). The color remains stable for about one hour and then fades slightly over a period of 24 hours.

Recoveries and Reproducibility of the Method.—Table I shows that the recoveries of antipyrine added to plasma were satisfactory. The mean recovery was 99.7 per cent with a standard deviation of 2.2. Each estimation was done in duplicate and the average error was less than 2 per cent.

Interfering Substances.—The following substances were tested for their interference: sulfanilimide, sulfadimidine, *p*-aminohippuric acid, pethidine, aspirin, aminopyrine, and phenobarbitone. Only the sulfonamides and *p*-aminohippuric acid interfered with the reaction.

TABLE I. RECOVERIES OF ANTIPYRINE ADDED TO PLASMA

ANTIPYRINE ADDED (μg)	ANTIPYRINE RECOVERED (μg)	PER CENT RECOVERED
4.0	4.2	105
7.5	7.3	97.5
8.0	8.0	100
10.0	9.95	99.5
12.0	12.0	100
15.0	14.6	97.5
16.0	15.8	99
20.0	19.7	98.5
22.0	22.2	101
30.0	30.6	102
35.0	34.0	97.5
40.0	39.4	98.5

Comparison of New Procedure With the Spectrophotometric Method.—Total body water was determined in 7 normal men after the intravenous injection of 1.5 Gm. antipyrine using the procedure of Soberman and associates[5] with regard to blood collection. Antipyrine was estimated both by the colorimetric method described and the spectrophotometric method of Brodie and coworkers[9] as modified by Davidsson and MacIntyre.[13] In each case the results obtained by the two methods differed by less than 2 per cent.

The Use of N-Naphthylethylenediamine Dihydrochloride as an Alternative Colorimetric Reagent.—N-naphthylethylenediamine dihydrochloride has been used as a reagent to estimate *p*-aminohippuric acid colorimetrically.[16] Since *p*-aminohippuric acid was found to interfere with the procedure described above, the use of N-naphthylethylenediamine dihydrochloride as a colorimetric reagent for antipyrine was investigated. Under the conditions of nitrosation described above, a 0.5 per cent aqueous solution of N-naphthylethylenediamine dihydrochloride produced a satisfactory color with antipyrine. A marked disadvantage of this reaction was the fact that the maximum color intensity developed only after 5½ to 6 hours. Fig. 6, B, shows the development of color over 2 hours. The fully developed color was approximately 2½ times as intense as that formed with 1-naphthylamine for any given concentration of antipyrine (Fig. 1, C).

The following modifications are suggested if N-naphthylethylenediamine dihydrochloride is to be used. Precipitation of plasma proteins and the conditions for nitrosation are the same as described above except that 1.0 ml. of protein-free filtrate is used. The blank is 1 ml. water and the standard is made up so as to contain 10 to 30 μg antipyrine per milliliter of water. After nitrosation has been completed, and excess nitrite destroyed by sulfamate, 1.0 ml. of a 0.5 per cent solution of N-naphthylethylenediamine dihydrochloride is added with mixing. Although color development is slow, it can be seen from Fig. 1, B, that the Beer-Lambert law is obeyed with amounts of antipyrine up to 30 μg after

one hour, so that readings may be taken then provided these are completed within 5 minutes. Since E_{max} of the color is at 594 mμ (Fig. 3, C) a 600 or 620 mμ filter should be used in the Evelyn photoelectric colorimeter.

Calculation.— $\frac{T}{S} \times \frac{20}{0.5}$ = micrograms antipyrine per milliliter of plasma, where T and S are the optical densities of the test and standard (20 μg antipyrine), respectively.

Work is in progress to define the conditions under which the color will be stable within a reasonable time and also to develop a micromethod for use in pediatrics.

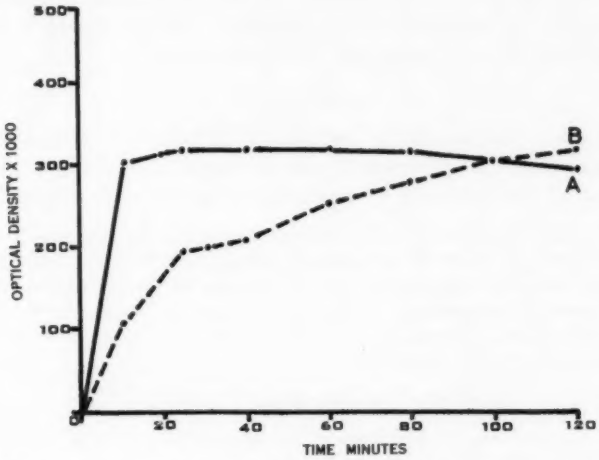


Fig. 6.—Development and stability of color. A = 20 μg antipyrine per 1.5 ml. using 10 per cent 1-naphthylamine in glacial acetic acid. B = 10 μg antipyrine per milliliter using 0.5 per cent aqueous solution of N-naphthylethylenediamine dihydrochloride.

DISCUSSION

The success of the reaction between antipyrine and 1-naphthylamine when adapted for colorimetric analysis depends upon two factors: (1) large concentrations of sodium nitrite are required for nitrosation of antipyrine, and (2) the absolute amount of acetic acid in the reaction mixture should be high since the intensity of the color increases as the concentration of acetic acid is raised (Fig. 5). In the method described the final concentration of acetic acid is 75 per cent. This has been achieved by modifying the protein precipitation procedure of Fujita and Iwatake[17] so that a relatively large volume of plasma is deproteinized by using small amounts of precipitating reagents. Thus, in the above procedure 0.75 ml. plasma will be contained in 1.5 ml. of protein-free filtrate. Further, the concentrations of the sodium nitrite and ammonium sulfamate reagents are high so that small quantities of these solutions are required.

A major criticism of the use of antipyrine to measure total body water has been its alleged binding to plasma protein.[5] Because of this fact, it was felt that compensatory binding in the tissues was necessary in order to arrive at a volume of distribution approximating that of total body water. Pace[18] remarked that a method which relied on a possible variable factor, such as the binding of antipyrine to tissue protein, was not a satisfactory one. However, more recent work[19] indicates that there is no binding of antipyrine to plasma protein. We have confirmed this observation using the technique of ultrafiltration described by Clegg[20] as modified by Mendelsohn.[21] Thus, the validity of the use of antipyrine for measuring total body water is further enhanced and because of the ease and accuracy of its determination in plasma and its ready availability it is suggested that at present it represents the most practical means of assessing total body water.

SUMMARY

A colorimetric method for the estimation of total body water using antipyrine is described. The procedure is simple, rapid, and accurate. The validity of the use of antipyrine to measure total body water is briefly discussed.

We are indebted to Professor H. B. Stein for allowing us the facilities of his department. We wish to thank Drs. T. H. Bothwell, B. Goldberg, and P. R. Levy for critizing the manuscript. The technical assistance of Miss A. Stransky of the Department of Chemical Pathology is gratefully acknowledged. Gratitude is expressed to Mr. A. M. Shevitz for photographing the figures. The expenses incurred in this study were defrayed by a grant from the University of the Witwatersrand.

REFERENCES

1. Hevesy, G., and Jacobsen, D. F.: Rate of Passage of Water Through Capillary Walls, Acta physiol. scandinav. **1**: 11-18, 1940.
2. Schloerb, P. R., Friis-Hansen, D. J., Edelman, I. S., Solomon, A. K., and Moore, F. D.: The Measurement of Total Body Water in the Human Subject by Deuterium Oxide Dilution, J. Clin. Invest. **29**: 1296-1310, 1950.
3. Prentice, T. C., Siri, W., Berlin, N. I., Hyde, G. M., Parsons, R. J., Joiner E. E., and Lawrence, J. H.: Studies of Total Body Water With Tritium, J. Clin. Invest. **31**: 412-418, 1952.
4. Glasstone, S.: Source Book on Atomic Energy, London, 1956, The Macmillan Company.
5. Soberman, R. J., Brodie, B. B., Levy, B. B., Axelrod, J., Hollander, V., and Steele, J. M.: Use of Antipyrine in the Measurement of Total Body Water in Man, J. Biol. Chem. **179**: 31-42, 1949.
6. Farber, S. J., and Soberman, R. J.: Total Body Water and Total Exchangeable Sodium in Edematous States Due to Cardiac, Renal or Hepatic Disease, J. Clin. Invest. **35**: 779-791, 1956.
7. Soberman, R. J.: Use of Antipyrine in Measurements of Total Body Water in Animals, Proc. Soc. Exper. Biol. & Med. **74**: 789-792, 1950.
8. Wintrobe, M. M.: Clinical Haematology, Philadelphia, 1956, Lea & Febiger.
9. Brodie, B. B., Axelrod, J., Soberman, R. J., and Levy, B. B.: The Estimation of Antipyrine in Biological Materials, J. Biol. Chem. **179**: 25-29, 1949.
10. Ikkos, D., Luft, R., and Sjogren, B.: Body Water in Patients With Acromegaly, J. Clin. Invest. **33**: 989-994, 1954.
11. Kraybill, H. F., Goode, G. R., Robertson, R. S. B., and Sloane, H. S.: In vivo Measurement of Body Fat and Body Water in Swine, J. Appl. Physiol. **6**: 27-32, 1953.
12. Zak, G. A.: The Role of the Nitrite Concentration in the Estimation of Antipyrine, J. LAB. & CLIN. MED. **49**: 623-625, 1957.
13. Davidsson, D., and MacIntyre, I.: Estimation of Antipyrine in Plasma, Biochem. J. **62**: 37P, 1956.

14. Delaville, M., Galli, A., Hiocco, and Lichtwitz, A.: Colorimetric Micro-estimation of Antipyrine and Its Applications to the Determination of Total Body Water, Ann. Biol. Clin. (Paris) **10**: 391-396, 1952. (Chem. Abst. **47**: 2240, 1953.)
15. Feigl, F., Costa Neta, Cl., and Silva, E.: Detection of Antipyrine and 1-Naphthylamine, Anal. Chem. **27**: 1319-1320, 1955.
16. Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., and Graber, M.: Renal Clearances of Substituted Hippuric Acid Derivatives and Other Aromatic Acids in Dog and Man, J. Clin. Invest. **24**: 388-404, 1945.
17. Fujita, A., and Iwatake, D.: Bestimmung des echten Blutzuckers ohne Hefe, Biochem. Ztschr. **242**: 43-60, 1931.
18. Pace, N.: Methods in Medical Research, Chicago, 1951, Year Book Publishers, Inc., vol. 4.
19. Shore, P. A., Brodie, B. B., and Hogben, C. A. M.: The Gastric Secretion of Drugs; a pH Partition Hypothesis, J. Pharmacol. & Exper. Therap, **119**: 361-369, 1957.
20. Clegg, R. E.: A Simple Method of Preparing an Ultrafiltrate, Chem. Analyst **38**: 87-88, 1949.
21. Mendelsohn, D.: A Micromethod for the Photometric Determination of Calcium in Serum With Ammonium Purpurate, South African J. M. Sc. **21**: 57-63, 1956.

IDENTIFICATION OF BARBITURATES BY PAPER CHROMATOGRAPHY

RUSSELL HILF, PH.D.,* GEORGE A. LIGHTBOURN, M.S.,** AND FRANK F. CASTANO, PH.D.
FORT BAKER, CALIF.

FOR the rapid determination of barbiturate levels in blood and urine, the ultraviolet absorption procedure of Goldbaum[1] has been used at this laboratory with excellent results.[2, 3] This procedure "is based on the observation that substituted barbiturates have an absorption band in strong alkali characteristic of one resonance form, while in solution at approximately pH 10, there is a different absorption band characteristic of another resonance form." The ratios of absorption calculated at various intervals in the ultraviolet range have been shown to be quite specific for the individual barbiturate. Since the identification of combinations of two or more barbiturates was not possible by the Goldbaum absorption procedure, other analytic procedures were investigated.

In a recent review, McBay[4] discussed the use of paper chromatography for the separation of barbiturates. The method of Algeri and Walker[5] was investigated for 19 commercially produced barbiturates. Rates of migration (Rf values) were obtained, and differentiation was made possible in most cases by the ability of various barbiturates to react to 3 different staining techniques. Confirmation of the identity of the particular barbiturate was accomplished by the elution of the stained area and characterization by ultraviolet spectral ratios.[1] Data were also obtained concerning the influence of atmospheric solvent concentration upon the rates of migration.

MATERIALS AND METHODS

In contrast to the original procedure,[5] descending paper chromatography was employed throughout. This was performed in a Chromatocab (Model A, Research Equipment Corporation, Oakland, Calif.) at room temperature. Whatman No. 1 filter paper (marked "for chromatography"), 18¼ by 22½ inches in size, was used for the separations. These large sheets were cut into strips 8 to 9 inches in width. Samples were spotted along a line approximately 3 inches from one end of the strip. Each sample was placed one inch apart from one another, and drying was aided by the use of a hair dryer. Standards were made up in ethanol to aid drying. The solvent was allowed to flow for 16 hours and it was most convenient to do this overnight. After migration, the solvent fronts were marked and the strips air dried.

From the Chemistry Division, 6th United States Army Area Medical Laboratory, Fort Baker, Calif.
Received for publication Nov. 25, 1958.
*Present address: Head, Cancer Endocrine Section, Squibb Institute for Medical Research, New Brunswick, N. J.
**Present address: 118 N. W. 14th Street, Miami, Fla.

Solvent.—The solvent system used was the same as reported previously.[5] Normal butanol saturated with 5 N NH_4OH was employed as the irrigating solvent. The aqueous phase, which was drawn off, was placed in beakers and set into the bottom of the chromatography tank to serve as the atmospheric solvent. Other combinations of butanol and ammonia did not produce better results.

Extraction of Barbiturates from Biologic Fluids.—Into a clean separatory funnel, 20 ml. of urine or 10 ml. of whole blood was placed. Samples employed in these studies were biologic fluids containing barbiturates in unknown quantities. The sample was acidified by the addition of 1 ml. of 10 per cent HCl. Twenty-five milliliters of chloroform was added and the barbiturates were extracted by shaking. The chloroform layer was removed and saved. This extraction procedure was repeated two more times, combining the chloroform extractions. The combined extracts were evaporated to dryness. The barbiturate residue was dissolved in 0.5 ml. ethyl ether and 20 μl (lambda) were spotted on the paper strips as described above.

Identification of Spots.—Four techniques were used for the location of the barbiturate after migration. These were the same procedures suggested by Algeri and Walker.[5] Excellent results were achieved using these techniques. Standards and unknowns were always spotted on separate sheets. Thus, all of the staining techniques could be performed, as well as obtaining triplicate determinations of Rf values.

1. Ultraviolet light: The air-dried sheets were first examined under ultraviolet light.* Fluorescence, observed as a bright purple spot upon a dark purple background, was noted.

2. Silver acetate: One dried sheet was irrigated with aqueous 1 per cent silver acetate solution. After 3 minutes, it was washed with water for one minute and immersed in a solution containing 0.1 per cent *sym.*-diphenylcarbazone in 95 per cent ethanol. The spots appear as dark brown to dark gray upon a gray-to-pink background. Excellent contrast was obtained using this procedure.

3. Lemaire's reagent: The second dried sheet was immersed in a solution containing 5 Gm. yellow mercuric oxide, 20 ml. concentrated sulfuric acid, and 100 ml. of water. After several minutes, the sheet was removed and washed in water to remove excess reagent. The sheet was then immersed in a solution containing 0.1 per cent *sym.*-diphenylcarbazone in 95 per cent ethanol. The barbiturates appear as dark purple spots upon a lighter purple background. This technique did not give as sharp a contrast as the silver acetate method, and the spots were not as clearly defined.

4. Potassium permanganate: The third dried sheet was immersed in a 10 per cent aqueous solution of potassium permanganate and washed thoroughly to remove any excess reagent. The spots appear as dark brown upon a light tan background. For the barbiturates that stain by this procedure, excellent contrast was noted.

Elution of Spots.—This procedure was employed with the silver-barbiturate salts. The spot was cut out of the paper and shredded into minute sections. These were placed in a 20 ml. beaker and 5 ml. of 10 per cent HCl was added. The contents were mixed and allowed to stand for 5 to 10 minutes. The supernatant was decanted and the procedure repeated. The combined supernatants were placed in a separatory funnel, and 10 ml. of chloroform was added. The chloroform used had been previously washed with 0.45 N NaOH.[1] The chloroform extraction was repeated and the extracts were combined and placed in a clean separatory funnel. The barbiturate was then extracted with 3.5 ml. of 0.45 N NaOH and the chloroform layer discarded. This solution was then placed in a 3 ml. silica cuvette and the absorbance determined in the ultraviolet range using a Beckman DU spectrophotometer. Blank solutions were prepared using blank paper and extracting in the same manner. The ratios of absorption are calculated and identification made.[1]

*United States Army Signal Corps Lamp, MX-1291/PAQ, 228 to 440 mμ, peak at 253.7 mμ.

RESULTS

The Rf values reported in these investigations were calculated from the sheets which were stained by the silver acetate technique. In the case of Evipal, which was the only one of the barbiturates investigated which did not form a silver salt, Rf calculations were made from the potassium permanganate stained sheet. The migrations were repeated several times and the Rf values reported are a mean of at least 3 determinations.

The Rf values obtained are recorded in Table I. The values obtained when migration was performed one day after the atmospheric solvent was placed in the tank showed a range from 0.47 for barbital to 0.77 for Pentothal. Also listed in Table I are migration values obtained when separation was carried out

TABLE I. MIGRATION VALUES AND STAINING REACTIONS OF BARBITURATES USING BUTANOL-AMMONIA SOLVENT

DRUG	Rf* 1 DAY	Rf* 5 DAYS	ULTRAVIOLET	SILVER ACETATE	LEMAIRE'S REAGENT	POTASSIUM PERMANGANATE
Alurate	0.63	0.74	purple spot	+	+	+
Amytal	0.73	0.83	-	+	+	-
Barbital	0.47	0.58	-	+	-	-
Butabarbital	0.65	0.76	-	+	+	-
Delvinal	0.57	0.68	purple spot	+	+	+
Dial	0.55	0.68	-	+	+	+
Evipal	0.71	0.81	-	-	-	+
Ipral	0.59	0.70	-	+	+	-
Mebaral	0.61	0.72	purple spot	+	+	-
Neonal	0.69	0.79	-	+	+	-
Ortal	0.76	0.85	-	+	+	-
Pentobarbital	0.71	0.82	-	+	+	black spot
Pentothal	0.77	0.87	+	+	+	+
Pernostan	0.69	0.80	-	+	+	+
Phanadorn	0.58	0.69	purple spot	+	+	+
Phenobarbital	0.50	0.61	-	+	+	-
Sandoptal	0.67	0.78	-	+	+	+
Seconal	0.76	0.85	-	+	+	+
Surital	0.72	0.83	-	+	+	+

*Rf values were calculated from separations performed 1 and 5 days after the addition of fresh atmospheric solvent.

5 days after the atmospheric solvent was placed in the chromatography tank. These results show a definite increase in Rf values, ranging from 0.58 to 0.87. It is interesting to note that this increase in Rf was approximately the same in each case.

Ultraviolet light clearly detected Pentothal, appearing as a greenish spot on a dark background. Four other barbiturates were also detected by fluorescence, appearing as a purple spot in contrast to the Pentothal. All of the barbiturates except Evipal stained with the silver acetate and *sym.*-diphenylcarbazone treatment. Table I also reveals that use of Lemaire's reagent stained all barbiturates except barbital and Evipal. Rf values were identical for both

staining procedures. When potassium permanganate was used as the staining procedure, only half of the barbiturates reacted. Pentobarbital gave a black spot as compared to the typical brown staining reaction of the others.

Noting that the Rf value varied with the length of time after atmospheric saturation, a study was conducted to determine the exact effect upon migration for various time periods. These results are shown in Table II. In general, the

TABLE II. INFLUENCE OF ATMOSPHERIC SOLVENT UPON Rf VALUES OF BARBITURATES

BARTITURATE	DAYS AFTER ATMOSPHERIC SOLVENT ADDED				NO ATMOSPHERIC SOLVENT
	1	4	6	7	
Dial	0.55	0.68	0.78	0.81	0.82
Barbital	0.47	0.58	0.67	0.71	0.75
Alurate	0.63	0.74	0.82	0.84	0.82
Pentobarbital	0.71	0.82	0.88	0.91	0.88
Pentothal	0.77	0.87	0.91	0.91	0.88

Rf value increased as the concentration of solvent in the atmosphere decreased. These values approach a maximum at 6 to 7 days after addition of the atmospheric solvent and were similar to the results obtained when no atmospheric solvent was used.

The elution procedure employing the silver-barbiturate salt appeared to be quite satisfactory for further identification of the barbiturate. Ultraviolet absorption spectra were obtained and recovery was good.

DISCUSSION

The migration values obtained at this laboratory agree with those reported by Algeri and Walker.[5] In addition, values are presented for 9 other barbiturates. Although the Rf values obtained were consistent and agreed with previously reported values, it was felt that any additional method of identification of these substances would be helpful in forensic studies. To this end, the elution of the migrated barbiturate was used and the ultraviolet characteristics obtained. The silver salt gave very distinct staining results and was easy to elute by the technique described. Another method which was used to eliminate any possible variation in Rf values from one time to another was the use of a standard chromatographed simultaneously. Migration ratios could be related to the Rf of the standard.

Some of the barbiturates could be distinguished by a combination of Rf value and ability to stain with certain reagents. The application of elution and ultraviolet characteristics presents an additional solution. Comparable results were reported by Allgen.[6] It would also seem possible that the eluted barbiturate could be further identified by microsublimation and melting point characteristics.

The increase in Rf value accompanying a decrease in concentration of the atmospheric solvent was investigated in order to determine the maximum time limitation before replacing the ammonia atmosphere. The data indicated that up to 4 or 5 days, the Rf values increased proportionately and no loss of accuracy was noted. However, beyond 5 days, the faster moving barbiturates did

not increase appreciably and equaled the Rf values when no atmospheric solvent was employed. On the other hand, the slower moving substances continued to increase and the differences in Rf values between barbiturates were lessened. Therefore, it seemed advisable that the atmospheric solvent be replaced every fourth day to give consistent results.

The use of paper chromatography is not convenient to the hospital or clinical laboratory because of the time involved and the techniques employed. For their purposes, establishing the presence and quantity of a barbiturate or barbiturates will usually suffice. In medicolegal practice, where differentiation of mixtures and positive identification is of importance, paper chromatography offers a distinct advantage.

SUMMARY

The use of paper chromatography for identification of barbiturates has been investigated. An elution procedure was devised and was demonstrated as being applicable for ultraviolet absorption spectra of the eluted barbiturate. The effect of atmospheric solvent concentration was also studied, migration increasing as atmospheric solvent concentration decreased. These results are discussed.

REFERENCES

1. Goldbaum, L. R.: Determination of Barbiturates, Anal. Chem. **24**: 1604, 1952.
2. Maher, J. R., and Puckett, R. F.: Identification of Barbiturates by Ultraviolet Absorption, J. Lab. & Clin. Med. **45**: 806, 1955.
3. Hilf, R.: Toxicology at the Army Area Laboratory, U. S. Armed Forces M. J. **9**: 843, 1958.
4. McBay, A. J.: Chromatographic Methods of Analysis Applied to Forensic Problems, J. Forensic Sc. **3**: 364, 1958.
5. Algeri, E. J., and Walker, J. T.: Paper Chromatography for the Identification of the Common Barbiturates, Am. J. Clin. Path. **22**: 37, 1952.
6. Allgen, L. G.: Separation and Identification of Barbiturates in Biological Material by Paper Chromatography, Scandinav. J. Clin. & Lab. Invest. **9**: 71, 1957.

A SIMPLIFIED AND EXACT METHOD FOR THE CONTROLLED SUBSTITUTION OF PROTEINS WITH I^{131}

NORMAN D. LEE, PH.D., AND RALPH WISEMAN, JR., M.S.
MEMPHIS, TENN.

WITH THE TECHNICAL ASSISTANCE OF ADA M. OLDHAM, B.S.

A NUMBER of methods have been devised for the substitution of insulin with iodine labeled with I^{131}. The suitability of such preparations for use in animals appears to have been based largely on the inability to detect diminished potency in the final product.[1-4] In this regard, however, rigorous and adequate testing has been a rarity; this topic has been discussed elsewhere and is the basis for another report.[5-6] One part of the problem lies in the uncertainties involved in the precise and reproducible preparation of proteins having specific and predictable levels of iodine substitution. Another aspect lies in the lack of information on the pertinent stoichiometric relations.

This report presents an extremely simple and rapid method for the exact preparation of pure aqueous iodine solutions of desired specific activity and their use in the iodination of insulin. The technique may be described as follows: (1) iodine generation using the required amount of I^{131}, (2) purification by solvent extraction, (3) specific activity determination by colorimetry (I^{127}) and radioassay (I^{131}), and (4) specific protein iodination. The use of this technique has permitted the reproducible preparation of insulin-I^{131} substituted with iodine up to levels as high as 4 iodine atoms per molecule on the average.

MATERIALS AND METHODS

Reagent grade chemicals were used without further purification and the I^{131} was obtained commercially. The crystalline zinc insulin was a gift from Dr. O. K. Behrens of the Lilly Research Laboratories. Radioisotope analyses were conducted with a conventional well scintillation counter-scaler system and the usual corrections applied as indicated. All insulin values were based on the micro-Kjeldahl analysis of reactant and product preparations.

I. Background Information.—A stock solution of elemental iodine was made by dissolving a carefully weighed sample in 1 N NaOH. Serial dilutions were prepared with 0.1 N NaOH, and 10 ml. aliquots were transferred to 40 ml. centrifuge cones. To each cone the following reagents were added in order: (1) 10 ml. of cyclohexane, (2) 2 drops of 3 per cent H_2O_2, and (3) 2 drops of 2 per cent ammonium molybdate. The contents

From the Radioisotope Service, Veterans Administration Medical Teaching Group Hospital, and the Department of Chemistry, University of Tennessee Medical School, Memphis, Tenn.

This work was partially supported by a grant-in-aid from Eli Lilly & Company, Indianapolis, Ind.

Received for publication Jan. 19, 1959.

were vigorously stirred with a motor-driven paddle and 1 N H_2SO_4 was added dropwise until no further brown I_2 color developed in the aqueous layer. The aqueous and organic phases were allowed to separate completely and an aliquot of the pink cyclohexane layer used for colorimetry.

Fig. 1 presents the absorption spectrum of a cyclohexane solution containing 0.5 μM of I_2 per milliliter. The instrument used was a Beckman DU spectrophotometer, although essentially the same results were obtained with a Model B; this latter instrument was used in the work presented in the remainder of this report. The color intensity was not observed to change over a 30-minute interval and decreased only 7 per cent over 24 hours.

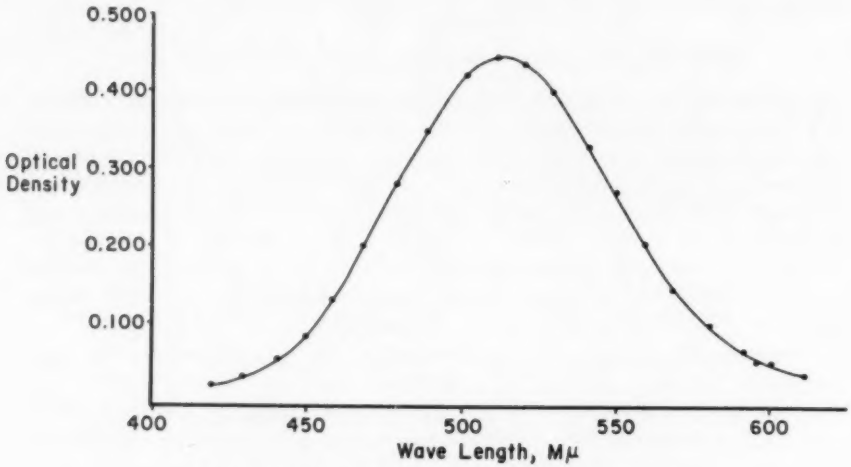


Fig. 1.—The absorption spectrum of I_2 in cyclohexane.

The I_2 concentration-optical density relationship was found to conform to Beer's law over the range tested, as high as 1 μM of I_2 per milliliter of cyclohexane, and the results of several determinations as described above are presented in Fig. 2. It was found that concentrations as low as 0.1 μM of I_2 per milliliter of cyclohexane could be reliably determined, and that concentrations as high as 5 μM per milliliter could be read by diluting aliquots of the intense pink organic layer with cyclohexane or benzene; with the latter diluent, however, the color stability was somewhat diminished as compared to the former.

Although these results were obtained on several occasions, it was felt desirable to check them by preparing I_2 by the following reaction:

$$5I^- + IO_3^- + 6H^+ \rightarrow 3I_2 + 3H_2O$$

This was done using KI, KIO_3, and H_2SO_4 and employing the same I_2 range stated above. For the direct method the optical density value for 0.5 μM I_2 per milliliter was 0.563 and for the KI–KIO_3 method it was 0.556; all other points agreed equally well.

Another question was that concerned with the efficiency of the extraction procedure. This was tested by generating I_2 using a KI solution to which a small amount of carrier-free I^{131} had been added. The optical density and I^{131} content of the cyclohexane were determined and the pink organic solution was extracted with H_2O to which a few drops of 1 N NaOH had been added. The organic layer became colorless and possessed a negligible amount of I^{131}. After it was removed, the colorless aqueous phase was radioassayed and

then extracted back into cyclohexane after acidification; the count rate and optical density of the second cyclohexane extract were also determined. The results are presented in Table I. They show quite clearly that both acid extraction into cyclohexane and alkaline extraction back into the aqueous phase are quantitative as shown by both the I^{131} and I_2 values.

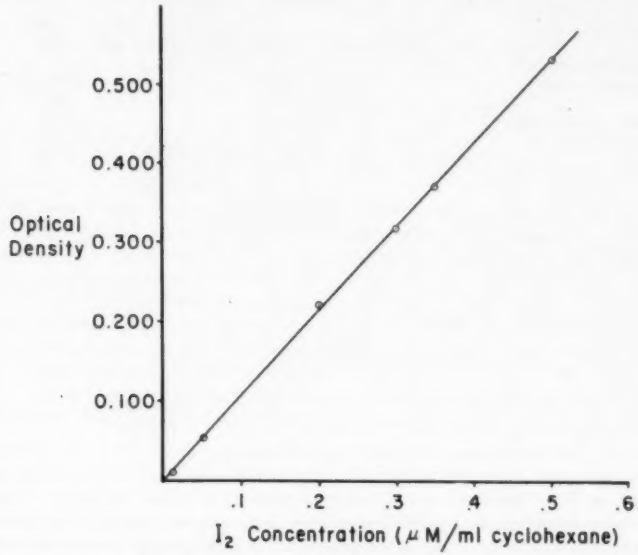


Fig. 2.—The Beer's law relationship of I_2 in cyclohexane.

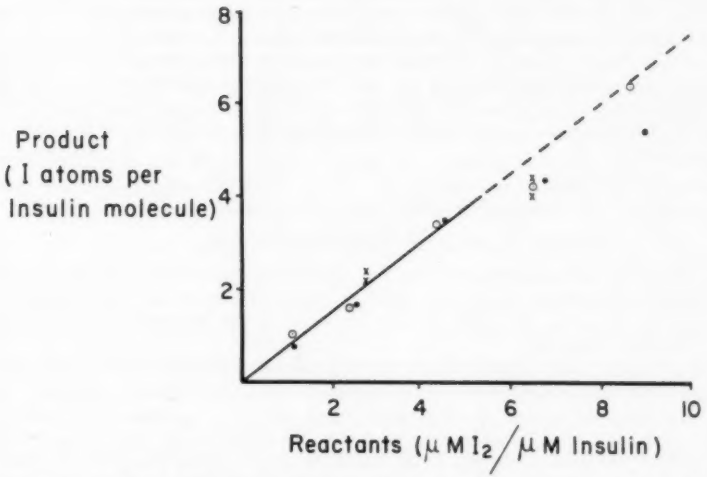


Fig. 3.—The relationship between the calculated ratio of reactants and the level of substitution of insulin-I^{131}.

TABLE I. THE EXTRACTABILITY OF I_2 INTO WATER AND CYCLOHEXANE
OPERATION

	I^{127} (O.D.)	I^{131} (C.P.M.)
$5I^- + IO_3^- + 6H^+ \xrightarrow[MoO_4 =]{H_2O_2} 3I_2 + 3H_2O$	--	4.65×10^6
↓ Acid extraction into cyclohexane		
→ Aqueous phase	--	0.1×10^6
Organic phase	0.517	4.45×10^6
↓ Alkaline extraction into water		
→ Organic phase	0.000	0.01×10^6
Aqueous phase	--	4.41×10^6
↓ Acid extraction into cyclohexane		
→ Aqueous phase	--	0.07×10^6
Organic phase	0.513	4.64×10^6

It had been found that the generation of I_2 from KI–KIO_3 sometimes deviated from anticipated results and this could best be correlated with the apparent contamination of the stock I^{131} solution. On a few occasions recoveries were as low as 85 per cent of theoretical or as high as 118 per cent. Generally such complications were completely corrected by oxidation with H_2O_2 in the presence of the molybdate catalyst; for this reason the catalytic oxidation step was routinely employed. However, stock I^{131} solutions were occasionally obtained which were grossly contaminated with a heavy flocculent precipitate and possessed a deep brown color. In such cases catalytic oxidation was not completely effective. It is for situations such as this that purification by solvent extraction and the conclusive determination of iodine recovery and specific activity are particularly valuable.

II. Preparation of the Iodinating Solution.—These findings provide a basis for preparing purified aqueous solutions of I_2 labeled with I^{131}. Such solutions can be designed according to specific needs with respect to quantity, concentration, and specific activity of the I_2–I^{131} and these specifications checked and controlled at the stage of the iodine solution in cyclohexane. The next step, extraction into the calculated volume of H_2O after alkalinization with NaOH, is extremely rapid and completely quantitative, the only precaution being to avoid the use of any more NaOH than absolutely necessary. The resultant solution may be used as required, addition of only a slight amount of H_2SO_4 being necessary to convert the various ionic forms of aqueous iodine to I_2,[7] about 5 minutes being necessary to bring this to completion. These latter steps as well as the succeeding steps should be done in the hood since some of the I_2 in the acidified aqueous solution may be lost by volatilization. Just prior to use the radioassay of an aliquot of this solution will permit an exact determination of the actual concentration of I_2 and, hence, of the amount used in the iodination.

III. Iodination Procedure.—At relatively low levels of protein substitution, less than 4 atoms per molecule, the following equation describes the reaction of insulin with I_2:

$$\text{Insulin} + I_2 \rightarrow \text{Insulin-I} + H^+ + I^-$$

Under the conditions described in this report, substitution occurs exclusively on the tyrosine residues.[8] Since there are 4 such residues per insulin molecule, each capable of being disubstituted, 8 iodine substitutions per molecule is the maximum that may be attributed to tyrosine iodination.

The use of the aqueous I_2 solution in labeling insulin is essentially the same procedure as that used commercially.[9] To the calculated volume of iodinating solution is added, with continuous mechanical stirring, the required amount of crystalline zinc insulin dissolved in 0.01 N HCl and the pH raised to 5.4 by the cautious addition of saturated Na_2HPO_4. For low levels of substitution decolorization of the brown I_2 solution is practically instantaneous and coincident with the isoelectric precipitation of the iodoinsulin; at higher levels complete blanching occurs more slowly but is complete in less than 5

minutes. Stirring is continued for a few minutes after which the iodoinsulin is washed by centrifugation several times with cold water to which a drop of saturated NaCl is added to minimize protein solubility.[10] In samples so treated, subsequent protein precipitation testing revealed no I^{131} unbound to protein.

IV. Example of Insulin Iodination.—Table II presents an actual iodination protocol designed for the substitution of insulin with approximately 2.2 iodine atoms per molecule,

TABLE II. PROTOCOL FOR THE SUBSTITUTION OF INSULIN WITH I^{131}

1. Materials	
A. KI	6.920 mg. } = 25 μM I_2
KIO_2	1.785 mg. }
B. I^{131}	4.32×10^7 c.p.m.
C. Insulin, crystalline zinc	0.975 μM (5.65 mg.)
2. I_2 Specific activity (cyclohexane step)	
A. I_2 analysis	29.5 μM, total
B. I^{131} analysis	3.73×10^7 c.p.m., total
C. Specific activity	$3.73 \times 10^7/29.5 = 12.6 \times 10^5$ c.p.m./μM I_2
	$3.73 \times 10^7/59.0 = 6.3 \times 10^5$ c.p.m./μA I
3. Iodinating solution, 10 ml.	$= 3.33 \times 10^7$ c.p.m., total
A. Total I_2	$\frac{3.33 \times 10^7}{3.73 \times 10^7} \times 29.5 = 26.3$ μM $I_2 - I^{131}$
B. I_2 concentration	$26.3/10 = 2.63$ μM I_2/ml.

on the average. The I^{131} stock was grossly contaminated, hence the aberrant values on I_2 and I^{131} recovery. The only effect this had was to reduce the anticipated I_2 specific activity from 17.3×10^5 c.p.m./μM to 12.6×10^5 c.p.m./μM; this would have no significant effect on the usefulness of the insulin–I^{131} in biologic experimentation and had no bearing on the final level of substitution. The cyclohexane was extracted into water and a volume equivalent to 2.63 μM of I_2 was used in the iodination; experience has shown that the I_2 volatilization losses, incurred subsequent to acidification and during the stirring prior to pH adjustment, were such as to necessitate the use of this amount of I_2 in order to obtain the level of labeling desired (Fig. 3). The results of 3 separate iodinations are presented in Table III. The excellent reproducibility of the product can be attributed to 3 factors: (1) rigorous control of I_2 volatilization losses by standardizing all aspects of procedure between acidification of the alkaline extract of the I_2-cyclohexane solution and adjustment of the pH of the reactants to 5.4; (2) the rapidity with which iodination takes place; and (3) substituting to levels less than 4 iodine atoms per molecule. The significance of this last factor is clearly seen in the values for the dashed portion of the curve of Fig. 3 and in the product values for preparations Nos. 4 and 5 in Table III.

DISCUSSION

The effective use of the method presented herein is simple, easy, and rapid, requiring no special equipment or unusual supplies. For its most precise application the main precautions are the avoidance of concentrated iodinating solutions (> 0.4 μM I_2 per milliliter) and the rigid standardization of such details of technique as time for I_2 development, solution volumes, and rapidity of stirring. Under these conditions the precise and predictable iodination of insulin with I^{131} of desired specific activity can be easily and quickly accomplished, with substitution occurring exclusively on the tyrosine residues. However, at levels of iodination greater than 4 iodine atoms per molecule, the conditions adopted in this laboratory lead to preparations somewhat more variable

TABLE III. SUMMARY TABLE ON THE PREPARATION OF 5 INSULIN I^{131} SAMPLES (SEE TABLE II)

PREPARATION NUMBER	REACTANTS							PRODUCT			
	I_2–I^{131}				INSULIN ADDED	REACTANT RATIO μM $I_2/\mu M$ INSULIN		I_2–I^{131} PROTEIN-BOUND		INSULIN	SUBSTITUTION LEVEL $\mu AI/\mu M$ INSULIN
	CALCULATED (ADDED)		ACTUAL (RECOVERED)			CALCULATED	ACTUAL				
	C.P.M. (1)	μM (2)	C.P.M. (3)	μM (4)	μM (5)	(2)/(5)	(4)/(5)	C.P.M. (6)	μA (7)	μM (8)	(7)/(8)
1	3.33×10^6	2.63	2.74×10^6	2.17	0.975	2.70	2.23	1.07×10^6	1.69	0.748	2.26
2	3.33×10^6	2.63	2.75×10^6	2.17	0.975	2.70	2.23	1.07×10^6	1.69	0.748	2.26
3	3.33×10^6	2.63	2.72×10^6	2.15	0.975	2.70	2.21	1.18×10^6	1.79	0.817	2.19
4	7.98×10^6	6.31	5.95×10^6	4.71	0.975	6.48	4.83	2.25×10^6	3.56	0.886	4.02
5	7.98×10^6	6.31	6.61×10^6	5.23	0.975	6.48	5.36	2.47×10^6	3.91	0.886	4.42

than at lower levels. Various factors may be responsible for this (the necessity for employing more concentrated iodinating solutions, longer reaction times, etc.), but it should also be remembered that the maximum number of substitutions at the primary reaction sites, the tyrosine residues, is being approached; consequently, one might expect such variability due to the apparently increasing role of possible side reactions.

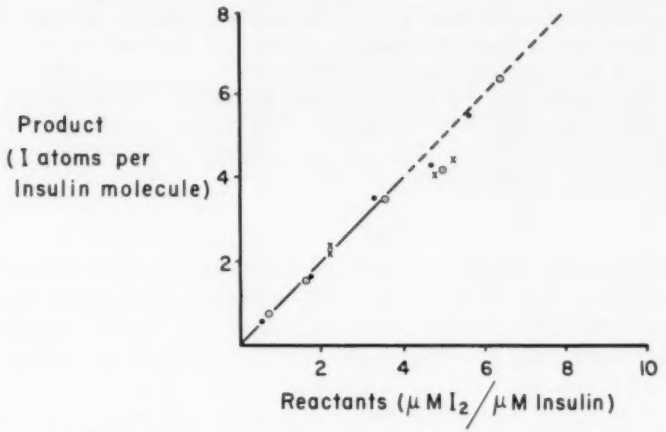


Fig. 4.—The relationship between the actual ratio of reactants and the level of substitution of insulin-I^{131}.

Another aspect of this method is concerned with its application to problems in stoichiometry. The curve in Fig. 3 represents the relationship between the calculated ratio of added reactants and the level of product labeling. The slope of this curve reflects the standardization features for I_2 volatilization losses that have been adopted in this laboratory; hence it demonstrates a working practical relationship, not necessarily a stoichiometric relationship. In order to investigate stoichiometry an exact determination of the actual amount of iodine participating in iodination is needed. This can be obtained by determining the total radioactivity of the product and all washes and relating this to the specific activity value for Step 2, C of the particular protocol. This figure then represents the actual amount of iodine participating in the reaction, the rest having been lost through volatilization. The data of Fig. 3 have been replotted, using the actual I_2 values in Fig. 4. It can be seen that the slope shows an exact and reproducible stoichiometric relationship up to the 4 iodine atoms per molecule level of substitution. This is the result to have been expected based on independent information on the iodination of proteins, particularly tyrosine residues.[8]

The final point of discussion lies in the significance of this method. These data have been presented not only as a method for protein iodination but also as a method for the simple and rapid preparation of pure aqueous I_2 solutions of known and standardized properties of specific activity and concentration.

Using this method a variety of iodoinsulin preparations have been made ranging in quantity from 3 to 30 mg. and in substitution from 0.5 to 7.25 iodine atoms per molecule; the upper limits of specific activity have not been explored since this will be limited by considerations other than those pertinent to this report (health physics and protein radiosensitivity). These preparations have been tested for hypoglycemic potency, and no losses were demonstrable until the substitution level began to approximate 2 iodine atoms per insulin molecule.[6] The data for insulin-I^{131} demonstrate the precise and reproducible manner in which such solutions can be used; it should be pointed out that the curve presented in Fig. 3 is based on 15 independent iodinations. The application of iodinating solutions prepared in this manner to other problems in protein iodination will, of course, be determined by the nature of the protein involved. However, the virtues outlined at the beginning of this paragraph should materially contribute to the solution of such problems.

SUMMARY

This report presents a simple, easy, and rapid method for preparing pure aqueous I_2–I^{131} solutions from KI–KIO_3 and carrier-free I^{131}. This method permits the exact determination and control of I_2 concentration and I^{131} specific activity. Data are presented showing the use of such solutions in the iodination of insulin.

REFERENCES

1. Stadie, W. C., Haugaard, N., and Vaughn, M.: Studies of Insulin Binding With Isotopically Labeled Insulin, J. Biol. Chem. **199:** 729, 1952.
2. Elgee, N. J., Williams, R. H., and Lee, N. D.: Distribution and Degradation Studies With Insulin-I^{131}, J. Clin. Invest. **33:** 1252, 1954.
3. Rose, S., and Nelson, J.: Studies With Radio-iodinated Insulin, Australian J. Exper. Biol. & M. Sc. **32:** 429, 1954.
4. Berson, S. A., Yalow, R. S., Bauman, A., Rothschild, M. A., and Newerly, K.: Insulin-I^{131} Metabolism in Human Subjects: Demonstration of Insulin-Binding Globulin in the Circulation of Insulin Treated Subjects, J. Clin. Invest. **35:** 170, 1956.
5. Lee, N. D.: Studies on Insulin Labeled With I^{131}, Ann. New York Acad. Sc. **70:** 94, 1957.
6. Lee, N. D.: Chemical Studies on Iodinated Insulin Preparations. (In preparation.)
7. Hughes, W. L.: The Chemistry of Iodination, Ann. New York Acad. Sc. **70:** 3, 1957.
8. Fraenkel-Conrat, J., and Fraenkel-Conrat, H.: The Essential Groups of Insulin, Biochim. et Biophys. Acta **5:** 89, 1950.
9. Gleason, G.: Personal communication.
10. Harington, C. R., and Neuberger, A.: Electrometric Titration of Insulin. Preparation and Properties of Iodinated Insulin, Biochem. J. **30:** 809, 1936.

A MOLD FOR PRODUCTION OF AGAR DIFFUSION PLATES

R. L. MILLER, B.S., AND ROBERT J. HECKLY, PH.D.
BERKELEY, CALIF.

A MOLDING apparatus was developed to facilitate the production of plates of agar gel for use with the Ouchterlony[1] double diffusion technique. The methods generally used require multiple operations to form wells in the agar and have the added disadvantage of dimensional variations among the replicate plates.

METHODS

Construction.—The mold (Fig. 1) consists of a sheet metal disk to which are affixed short tubular plugs arranged and spaced to suit the work at hand. Three legs near the edge of the disk support the assembly and maintain the bottoms of the plugs at a desired height (2 to 3 mm.) above the bottom of the Petri dish. A fourth peg near one of the legs serves as a point of reference for identifying individual wells. Stub wires are arranged and spaced on a second disk for ready insertion and occlusion of the bores of the tubular plugs when the two parts of the mold are enmeshed.

Operation.—In the casting operation, a desired amount (20 to 50 ml.) of molten agar is poured into the Petri dish and the mold assembly is placed in position. After the agar has solidified an air passage to the bottom of the wells is provided by removing the upper disk with its attached occluding wires from the lower disk. Finally, by raising the lower plate, the plugs are lifted out of the agar. This leaves wells in the agar having straight perpendicular sides and clean-cut unfractured edges. If the plugs are not vented as described above, the mold is removed with greater difficulty and frequently the upper edge of the agar around the well is torn. Fig. 1 illustrates a typical mold currently in use at the Naval Biological Laboratory and an example of an agar diffusion plate produced by it. The results of studies using molds for 5, 7, 13, and 15 wells in various patterns with the wells at distances from 15 to 25 mm. apart indicate that there is no demonstrable difference between replicate plates.

From the Naval Biological Laboratory, School of Public Health, University of California, Berkeley, Calif.

This work was sponsored by the Office of Naval Research and the Bureau of Medicine and Surgery, United States Navy, under a contract between the Office of Naval Research and the Regents of the University of California.

Opinions expressed in this report are not to be construed as reflecting the views of the Navy Department or of the Naval Service at large (Article 1252, U. S. Navy Regulations, 1948). Reproduction in whole or in part is permitted for any purpose of the United States Government.

Received for publication Jan. 23, 1959.

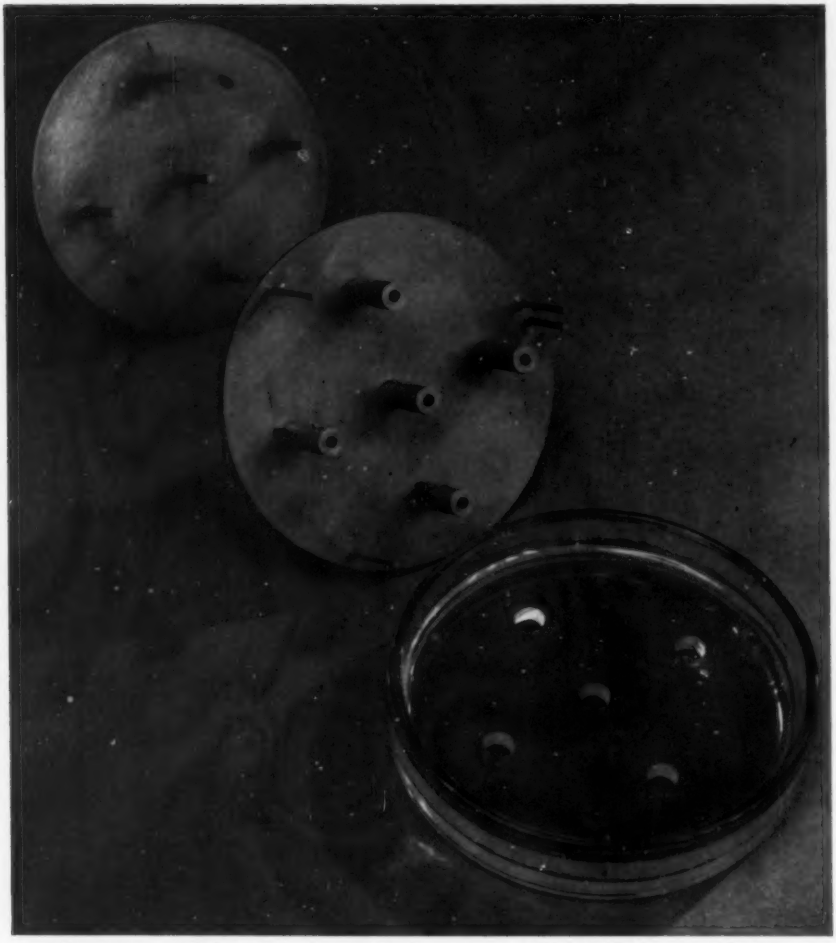


Fig. 1.—An exploded view of a molding apparatus and a finished agar diffusion plate ready for use. The pins on the upper disk occlude the bores of the plugs on the lower disk during the casting operation and on removal provide a vent to facilitate removal of the plugs from the agar.

SUMMARY

Uniform agar diffusion plates were easily prepared using molds with precisely spaced plugs. Venting the plugs with removable stub wires facilitated the separation of the molds from the agar plates. The time saved by using the molds for the preparation of agar diffusion plates, instead of the conventional method of locating individual wells by hand, has justified the cost of manufacturing the molds.

REFERENCE

1. Ouchterlony, O.: Antigen-antibody Reactions in Gels, Acta path. et microbiol. scandinav. **26**: 507-515, 1949.

One Manufacturer's Opinion

A Welcome Borrowing

Observing the success of quality control in industry, professional groups are asking why quality control techniques should not be applied to other areas. Recent events point up demands by leading scientists for this application—especially to those areas dealing with the safeguarding of human life.

Aside from the particular product or service offered by a manufacturer, long-term success is, after all, dependent on the guarantee of reliability and quality offered the consumer. Knowing this, industry has developed rigid quality control over production. Extensive testing, taking weeks or months, is undertaken before release of a product run. This testing serves a dual purpose. First, efficiency in manufacturing is maintained and guarded; secondly, the product or service offered is kept at a standard to satisfy the discriminating public.

In the clinical laboratory, advances made in specific instrumentation, methods, technique, and diagnostic reagents have, until recent years, greatly exceeded the overall quality control practiced over biochemical determinations.

It is not surprising that modern advances and antiquated control are functioning side by side in laboratory testing. *Adequate control measures have not been available.*

Now, two major steps have been taken to fill this void. First, the application of statistical measures of precision and of accuracy, and the routine use of the specific deviation yardstick are becoming widespread *in revealing* laboratory efficiency problems. Secondly, the common usage of two important standards-in-serum for biochemical testing, Versatol (normal) and Versatol-A (abnormal), with known absolute values in the normal and abnormal range, meet the demands of advanced laboratory analyses.

Combined with technical skill, these safeguards over laboratory testing add welcome new chapters to accuracy in clinical chemistry.

Versatol™

Versatol-A™

Quality control in clinical chemistry featured at scientific meeting

Clinical Pathologists, Chemists Eye Application of Industrial Advances to Laboratory

New York, N. Y., Nov. 12, 1958. Interest in clinical laboratory quality control was heightened today when, the second time this month, this subject was covered at the Symposium on Laboratory Instrumentation at the Hotel New Yorker. Stephen J. Koziol of Warner-Chilcott, in a paper on "Recent Developments in Increasing the Efficiency of Clinical Analyses", stated the approach used by his firm:

"The third ... part deals with the objective standards and the objective controls which may be applied by the conscientious laboratory worker—to assure that neither human fatigue nor instrument fatigue are giving rise to clinically invalid results. Reference standards—closely duplicating both normal and pathological clinical chemistry specimens ... have been developed to aid quality control within the clinical laboratory itself."

Two reference standards, Versatol and Versatol-A, which closely duplicate both normal and pathological clinical chemistry specimens have been developed to aid quality control within the laboratory itself.

Earlier, in this same Symposium, pioneers in microchemistry recommended Versatol and Versatol-A for the *standardization* of the newest microchemical methods.

Quality Control in Clinical Chemistry was a featured presentation earlier this month at the American Association of Clinical Pathologists meeting in Chicago. The use of freeze-dried reagents *like* Versatol and Versatol-A was recommended for routine daily control.

GENERAL DIAGNOSTICS DIVISION **WARNER-CHILCOTT** DIV. MORRIS PLAINS, N.J.

Do You Encounter Bone Tumors or Tumor-Like Conditions?

This New Edition Is a Practical Working Guide to Their Recognition and Management

Just Published! 2nd Edition

BONE TUMORS

by

Louis Lichtenstein, M.D.

By LOUIS LICHTENSTEIN, M.D., Chief Pathologist, General Medical and Surgical Hospital, Veterans Administration Center, Los Angeles; Fellow, New York Academy of Medicine; Professor Extraordinario, National University of Mexico; Consultant in Bone Tumors, Tumor Tissue Registry of California Medical Association Cancer Commission. Just Published. 1959, 2nd edition, 402 pages, 6 3/4" x 9 3/4", 220 illustrations. Price, $12 00

If you encounter bone tumors or tumor-like conditions from time to time and must be able to recognize them and treat them appropriately you'll find the new 2nd edition of BONE TUMORS particularly valuable. Unusually well illustrated with X-ray pictures and photographs, Dr. Lichtenstein's book is a clear, concise presentation of the latest clinical, radiologic and pathologic concepts on benign and malignant tumors. Emphasizing accurate diagnosis as a basis for appropriate treatment, this new 2nd edition incorporates all of these features:

New roentgenograms and photomicrographs

Virtually every chapter rewritten to include all of the recent advances in the field

A new introductory chapter directed to clinicians and pathologists as a guide to appropriate management of bone lesions that may be tumors

A new chapter on benign osteoblastoma; unusual chondroid tumors and tumors of periosteal origin

A discussion on tumors of synovial joints, bursae and tendon sheaths added to the appendix as collateral material

The section on non-neoplastic lesions of bone which may be mistaken for tumors has been amplified

Based on the author's broad personal experiences, this book stands as one of the truly authoritative books in the field. Be sure to examine a copy and see for yourself how it can help you.

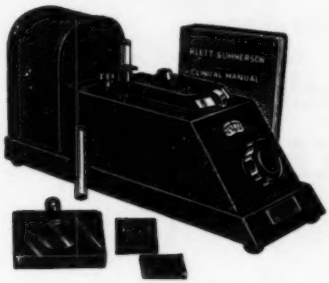
Purchase Any of Three Ways:
At Your Favorite Bookstore;
From Our Personal Sales Representative;
Or Order on 10 Day Approval From

C V M **The C. V. Mosby Company**
3207 Washington Boulevard • St. Louis 3, Missouri

KLETT SCIENTIFIC PRODUCTS

Klett-Summerson Photoelectric Colorimeters, Colorimeters, Nephelometers, Fluorimeters, Bio-Colorimeters, Comparators, Glass Standards, Glass Cells, Klett Reagents

THE KLETT-SUMMERSON
PHOTOELECTRIC
COLORIMETER



Developed with the requirements of routine colorimetric analysis particularly in mind, but has found application in a variety of unusual analytical procedures as well. No logarithm tables or "calibration curves" on semilogarithmic paper required. The concentration of unknown is obtained directly from the scale reading by simple calculation.

$205

KLETT
BIO-COLORIMETER

The visual colorimeter illustrated presents a number of outstanding features making it ideal for clinical or research work. Built on the most modern mechanical and optical principles it is widely accepted for its accuracy and durability.

$165

Klett MANUFACTURING CO.
179 EAST 87th STREET, NEW YORK, N. Y.

FOR MEN AND WOMEN!
A WORTHWHILE CAREER IN
LABORATORY TECHNIQUE

THE GRADWOHL SCHOOL OF LABORATORY TECHNIQUE is an ethical school, manned by competent ethical physicians and technologists and enjoys a high rating among the medical profession . . . Graduates placed in desirable positions since 1920. Gradwohl graduates are recognized as expert technicians.

Course includes—Clinical Microscopy; Hematology and Blood Grouping; Serology; Applied Bacteriology; Basal Metabolism; Blood Chemistry; Electrocardiography; Parasitology; Tissue Cutting and Staining.

ENROLL NOW for priority. 12-month course; 6-month internship Classes begin Jan., March, June, July, Sept., Oct.

39th Successful Year

GRADWOHL SCHOOL OF LABORATORY TECHNIQUE

3514 Lucas Ave. St. Louis 3, Mo.

...take the step to...

ONE COMPLETE SOURCE FOR ALL YOUR RESEARCH BIOCHEMICALS NEEDS

A COMPLETE SELECTION OF MORE THAN 200 NUCLEOPROTEINS AND DERIVATIVES

Typical Derivatives

Adenosine Triphosphate	Uridylic Acid
Cytidine	Uridine
Cozymase	2, 6 Diamino Purine Sulfate
Coenzyme I, II, A	8 Aza Guanine
Cytosine	8 Aza Thymine
6 Mercaptopurine	Glucose-6-Phosphate

Write for New Catalog
July, 1959, More than
2,500 Items. Write Dept. 109

NUTRITIONAL
BIOCHEMICALS
CORPORATION

21010 MILES AVE. • CLEVELAND 28, OHIO

Increase the Range of Your Office Work

Improve the Accuracy of Your Diagnosis

Williamson

Practical Use of the OFFICE LABORATORY and X-RAY

(Including the Electrocardiograph)

1957
322 pages
6⅞" x 10"
illustrated
Price, $10.75.

A knowledge and understanding of the scope and limitations of your laboratory equipment and x-ray *and the proper interpretation of tests and films* are the keys to successful use of your equipment. With these keys you can increase the accuracy of your diagnosis and broaden the scope of your office practice.

In clear, understandable language, "Practical Use of the OFFICE LABORATORY and X-RAY" shows how you can utilize your equipment to maximum benefit—how you can make your laboratory and x-ray valued tools *to answer specific questions designed to confirm or deny your original findings* and not merely instruments to make "routine test reports."

Each procedure discussed can be done by a home-trained technician in any office with a minimum of basic equipment. Carefully-organized and well-illustrated, this practical guide discusses in detail each specific test, the equipment to be used, the technique, the interpretation of results and the errors most commonly made. The useful information you can gain from your office laboratory and x-ray is far more extensive than many physicians achieve with such equipment.

By PAUL WILLIAMSON, M.D.

Date_____

The C. V. MOSBY Company

3207 Washington Boulevard, St. Louis 3, Missouri

Gentlemen: Please send me Williamson, "PRACTICAL USE OF THE OFFICE LABORATORY AND X-RAY" priced at $10.75 on 10 day approval. I understand if I am not completely satisfied I can return the book within 10 days with no charge or obligation. If remittance is enclosed, publisher pays the mailing charge.

☐ Payment enclosed ☐ Charge my account

Dr. _____

Street _____

City _____ Zone _____ State _____

LAB-8-59

Mod. 401-A

Write for descriptive
Bulletin No. 406
also for free copy of 12-page
Introduction to Clinical
Photoelectric Colorimetry.
180-page Reference Book
available separately, price $8.00.

PHOTOVOLT Corp.
95 Madison Ave. ● New York 16, N. Y.

TRANSAMINASE

SGO—T and SGP—T

Glutamic Oxaloacetic and Glutamic Pyruvic Transaminase according to Cabaud, Wroblewski et al.,
(Journal of Clinical Pathology, 1956)

with

LUMETRON Clinical

PHOTOELECTRIC COLORIMETER

also 85 determinations including

Albumin
Amino Acids
Amylase
Bilirubin
Blood Volume
Bromide
Bromsulphalein
Calcium
Carbon Dioxide
Chloride
Cholesterol
Cholesterol Esters
Creatine
Creatinine
Cyanmethemoglobin
Ethyl Alcohol
Fibrinogen
Galactose
Gamma Globulin
Globulin
Glucose
Hemoglobin
Hydrogen Ion
Icteric Index
Iron
Lactic Acid
Lead
Lipids
Magnesium
N.P.N.
Phenols
P.S.P.
Phosphatase
Phosphorus
Potassium
Protein
RBC Count
Salicylates
Sodium
Sulfa Comp
Thiocyanate
Thymol Turb.
Urea Nitrogen
Uric Acid
Urobilinogen
Vitamin A
Vitamin C
Zinc Sulphate
17-Ketosteroids

worthington

AN IMPORTANT NOTICE TO USERS OF GLUCOSTAT PAST, PRESENT OR FUTURE

Our control laboratory recently obtained results with a routine check in Glucostat which failed to give a linear response with standard glucose solutions.

This was the first time since the reagent was formulated three years ago that it failed to perform satisfactorily.

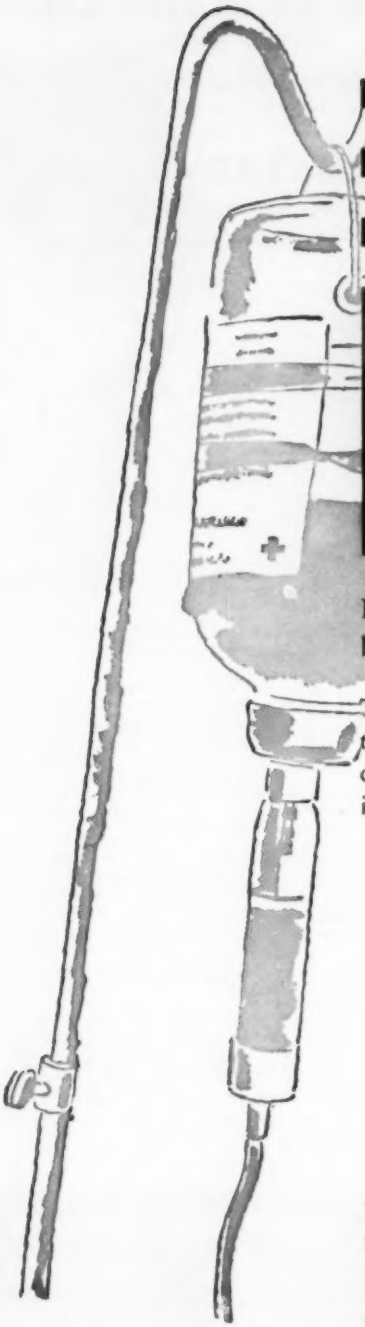
After checking all possibilities of error, it was discovered that the reservoir of distilled water had recently been fitted with gum rubber tubing. When the water used to make up the reagent was taken directly from the still the results were normal. Evidently, something in the tubing inhibited the glucose oxidase-peroxidase reaction.

We suspected the tubing since one of our customers had told us some time ago that a deproteinizing reagent stored in an automatic pipette fitted with rubber connections caused interference with this reaction.

We therefore caution all users about this potential hazard.

If you have not yet tried Glucostat for blood or urine glucose, or, any other quantitative specific glucose determination, write:

worthington
BIOCHEMICAL CORPORATION
FREEHOLD NEW JERSEY



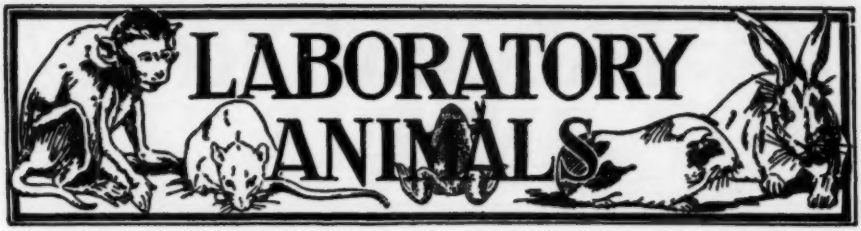
MORE THAN MEETS THE EYE . . .

Physicians know that in every pint of blood lie vital hidden treasures.

The AMERICAN RED CROSS knows this, too. Through the Blood Program, licensed by the National Institutes of Health, it provides

- **whole blood to hospitals served by the program**
- **blood for national emergencies**
- **blood derivatives to physicians and hospitals . . . serum albumin, gamma globulin, fibrinogen, fresh-frozen plasma and packed red cells**
- **crude fractions for research**

Encourage donors to expand the availability of life's most precious fluid by giving blood wherever there are facilities for receiving it.



(See also pages 32, 33)

HEMLOCK HOLLOW FARM
BREEDERS OF LABORATORY ANIMALS

LABORATORY ANIMALS
FOR SCIENTIFIC & MEDICAL USES

RATS • • MICE
GUINEA PIGS • • RABBITS
Clean, Ventilated up to date Buildings-
Industrial Washing Machine, Steam
Sterilizer - Regular Testing of Stock
for Detection of Disease.
Send for brochure

HEMLOCK HOLLOW FARM
414 BLACK OAK RIDGE ROAD WAYNE NEW JERSEY

LABORATORY ANIMALS
since 1929

Albino Swiss-W Mice
Albino Rats from Wistar Strain
Albino Guinea Pigs
Albino NZ Rabbits
Hamsters

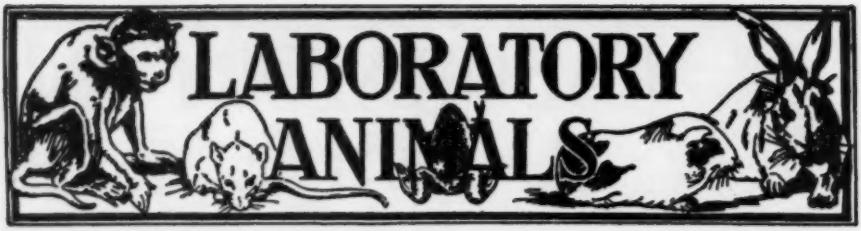
* stainless steel watering tubes for laboratory animals BENT or STRAIGHT

ALBINO FARMS
P. O. Box 331
RED BANK, NEW JERSEY

MICE
RATS
RABBITS
GUINEA PIGS

CHERRY HILL FARMS
P.O. BOX 840, CAMDEN 1, N. J.

RABBITS GUINEA PIGS
Developed and housed to insure best results in all types of tests
Continuous supply and prompt shipment FOB Indianapolis
PAUL HAMM RABBITRY R.R. No. 4 TUcker 1-2188 Greenwood, Ind.



(See also pages 31, 33)

LIVE FROGS for PREGNANCY DIAGNOSIS

Rana pipiens

Year 'round supply of selected large, healthy, male frogs for latest pregnancy test (99% accuracy). Cost negligible. Negative test animals can be used repeatedly. **Results in less than two hours.** Prompt shipment anywhere, by air to distant points. **Prices include screened shipping crates and boxes.**

Frogs. With complete directions for use and care. Dozen	**$ 7.50**
Laboratory Tank with Cover	**7.00**
Isolation Jar, 5" x 7" with weighted metal cover	**3.50**
Frog pregnancy test kit, complete, 14 items including 12 live frogs	**35.00**

Est. NYSG **1919**

NEW YORK SCIENTIFIC SUPPLY CO. - - - - 28 West 30th St., New York 1, N. Y.

Headquarters for Live Frogs

Male Frogs for Pregnancy Tests

One dozen $3.50, or $3.00 per dozen for 2 dozen or more. Mealworms (food), thousand $3.35. Postage extra. Frog Bulletin and Scientific Supply Catalog on request.

QUIVIRA SPECIALTIES CO.

4204 W. 21st Street -:- -:- -:- Topeka 32, Kansas

ALBINO RATS & MICE, RABBITS & GUINEA PIGS

"Our 44th Year—Time Proved Dependability"

RESEARCH SUPPLY CO.

2436 West York St. Philadelphia 32, Pa.

If you use CASTRATE RATS in your research and the rats come from any of the Madison sources, write for information about our CASTRATE SERVICE. We can save you time and money.

ZEIMET BIO-FARMS

Route 4, Box 372
Madison 4, Wis.
CHerry 9-4515

GUINEA PIGS

FROGS

Special Males for Pregnancy and Pre-operative Diagnosis.

Fast Service. All orders shipped via air same day received. Live delivery guaranteed.

Write for handbook giving full information

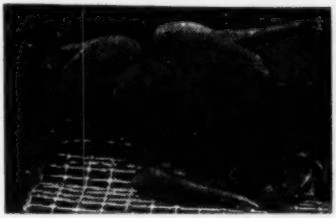
E. G. STEINHILBER & CO., INC.

102 Josslyn Avenue (Est. 1922) Oshkosh, Wis.

(See also pages 31, 32)

SINCE 1945

X-L FEMALE FROG PREGNANCY TEST



Highest Year-Round Accuracy—
Economy—Speed

SUGGESTED $15. TRIAL ORDER

2 X-L female frogs (each frog may be used repeatedly for years)
1—6-inch Isolation Jar
Reagents for 100 tests

Complete instructions included.
Safe delivery guaranteed.

JAY E. COOK, Importer
Cockeysville, Maryland, U.S.A.

HAMILTON LABORATORY ANIMALS, INC.

Specialist in breeding of HLA Albino Rats and HLA Albino Swiss Mice. Prompt and efficient service. Dependable animals.

7120 Pippin Road, Cincinnati 39, Ohio

DAN ROLFSMEYER CO.

ALBINO RATS **SWISS (ALBINO) MICE**

Price List on Request

Route 3, Syene Road, Madison 5, Wis. **PHONE ALpine 6-6149**

Live Male Rana Pipien Frogs, Direct from the Farm

Be sure of year round supply, at lowest prices and fastest service available. Orders delivered within 36 hours after we receive them.
Male Rana Clamitan Frogs and Buffo Americanus Toads available during summer months.
Male Buffo Marinus Toads available, the year around.
Animals guaranteed free of disease and to arrive in perfect condition.
Laboratory Manual 85 pages with illustrations. A system of pregnancy testing using Male Toads and Frogs, by Edward H. Hon, M.D., of Yale University.
Also reagents used in conducting pregnancy tests.

For information and prices, Phone or write:

J. R. SCHETTLE FROG FARM, INC. Stillwater, Minnesota

TACONIC FARMS, Inc.

Germantown, New York

SWISS MICE *Booklet Sent on Request*

The Journal of Laboratory and Clinical Medicine

The Official Publication of the Central Society for Clinical Research

EDITOR
William D. Robinson, M.D.

ASSOCIATE EDITOR
William H. Beierwaltes, M.D.

University Hospital
University of Michigan Medical School
1313 Ann Street
Ann Arbor, Michigan

PUBLISHED BY THE C. V. MOSBY COMPANY, 3207 WASHINGTON BLVD.
ST. LOUIS 3, U. S. A.

Published monthly. Subscriptions may begin at any time.

Editorial Communications

The Journal of Laboratory and Clinical Medicine is published under the editorial direction of a Board of Editors appointed by the Central Society for Clinical Research. Manuscripts describing original observations in the broad fields of clinical investigation and clinical pathology are invited. Society affiliation will not prejudice or influence editorial decisions.

Manuscripts.—All correspondence relating to the publication of papers should be addressed to the editor. Manuscripts must be typewritten, double or triple spaced, on only one side of the paper. They should be organized under the following general headings: Introduction, Materials and Methods, Results, Discussion, and Summary. Academic degrees of authors should be given. Bibliographic references should include the titles of papers and should conform to the following style: Author, Title, Journal, Volume, Inclusive Pages, Year. Only one copy, the original, should be submitted.

Neither the editor nor the publisher accepts responsibility for the opinions of contributors, nor are they responsible for other than editorial statements.

Illustrations.—A reasonable number of halftone illustrations will be reproduced free of cost to the author, but special arrangements must be made with the editor for color plates, elaborate tables, or extra illustrations. Copy for zinc cuts (such as pen drawings and charts) should be drawn and lettered in India ink or black typewriter ribbon (when the typewriter is used). Only good glossy photographic prints or original drawings should be supplied for halftone work.

Reprints.—Reprints must be ordered directly from the publishers, The C. V. Mosby Company, 3207 Washington Boulevard, St. Louis 3, Mo., who will send their schedule of prices. Individual reprints of an article must be obtained through the author.

Business Communications

Business Communications.—All communications in regard to advertising, subscriptions, change of address, etc., should be addressed to the publishers, The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo.

Subscription Rates.—United States and its Possessions $13.00, Students $6.50; Canada, Latin-America, and Spain $13.50, Students $7.00; Other Countries $14.50, Students $8.00. Single copies, $2.00 postpaid. Remittances for subscriptions should be made by check, draft, post office or express money order, payable to this Journal.

Publication Order.—The monthly issues of this Journal form two semiannual volumes; the index is in the last issue of the volume—in the June and December issues.

Change of Address Notice.—Six weeks' notice is required to effect a change of address. Kindly give the exact name under which a subscription is entered, and the full form of both old and new address, including the post office zone number.

Advertisements.—Only articles of known scientific value will be given space. Forms close first day of the month preceding date of issue. Advertising rates and page sizes will be furnished on application.

Bound Volumes.—Publishers' Authorized Bindery Service, 5811 West Division Street, Chicago 51, Illinois, will quote prices for binding complete volumes in permanent buckram.

SPECIALIZED SERVICES 7 DAYS A WEEK *to Laboratories all over North America*

TOXICOLOGY & MISCELLANEOUS CHEMISTRIES

Lead • Arsenic • Transaminase
Lipids • Antistreptolysin
Hemoglobin Identification
Proteins by Electrophoresis
Serum Iron • Iron Binding Capacity • Paternity Tests
Copper • Magnesium

ENDOCRINE ASSAYS

Aldosterone • Catecholamines
Serotonin • 17-Ketosteroids and Fractionations • Protein-Bound Iodine • Estrogens
Butanol Extractable Iodine
Pregnanediol • Pregnanetriol
Corticosteroids • Gonadotropins

"The Bulletin of Laboratory Medicine" is published monthly by Biochemical Procedures to keep physicians and laboratorians abreast of current developments in the clinical laboratory field. Write for a complete set.

Please write for Fee Schedule & Mailing Containers

BIOCHEMICAL PROCEDURES

12020 Chandler Boulevard
North Hollywood, California

"The Laboratory for Laboratories"

REFERRED BIOCHEMICAL ANALYSES

Electrophoresis for Protein Fractionation, Lipoprotein Fractionation, Abnormal Hemoglobins; **Catecholamine** determinations for adrenaline and noradrenaline; **Transaminase** levels; **Hormone** Assays; **Protein-Bound Iodine** determinations; **Toxicological** Assays; Antistreptolysin-O Titers; and other difficult and time-consuming tests.

Services available nationally by Air Mail. Shipping containers with vials available on request

Write for: List of Services, Literature, and Containers

CHEM-TECH LABORATORIES

ANALYTICAL - CLINICAL - RESEARCH

2361-2381 SOUTH ROBERTSON BLVD., BEVERLY HILLS, CALIFORNIA

S. I. DULKIN, PH.D. OTTO E. LOBSTEIN, PH.D

Changing Your Address?

WHEN YOU MOVE, PLEASE—

(1) Notify us to change your address—allow us six weeks to make the change.
(2) Mention the name of this Journal. (We publish twelve periodicals.)
(3) Give us your old address. If possible, return the addressed portion of the envelope in which we sent your last copy.
(4) Give us your new address—complete—including the Postal zone number.
(5) Please print your name and address.

Thank You!

Circulation Department, The C. V. Mosby Company, Publishers, 3207 Washington Blvd., St. Louis 3, Mo.

A Comprehensive, Readable Synthesis on Hematology

Miale

LABORATORY MEDICINE —HEMATOLOGY

While other references in clinical pathology present in one volume a survey of all three broad fields—hematology, chemistry and microbiology—the new Miale book, LABORATORY MEDICINE—HEMATOLOGY is the first truly comprehensive evaluation of the ENTIRE FIELD of hematologic diagnosis beginning with the basic concepts of hemopoiesis and cell survival and progressing to a detailed presentation of the entire field.

The first in a series of three separate volumes on laboratory medicine, this is a detailed investigation of virtually every important aspect of hematology. You'll find within the covers of this volume a complete atlas of the morphology of blood cells complete with highly-instructive photomicrographs (not idealized drawings) visually describing what one actually sees in the microscope accurately authenticated at the standard magnification and with the usual stain. You'll consult the chapter on the myeloproliferative syndromes often since it is one of the few really complete syntheses of this puzzling group of diseases. You'll appreciate the book's thoroughness and timeliness—covering as it does all current literature up to publication. The chapter on blood coagulation is the most complete and up-to-date discussion available outside of special works, and Dr. Miale's original studies on vascular pseudohemophilia make this one of the most comprehensive and highly-essential treatises on hematology available today.

By JOHN B. MIALE, M.D., Professor of Pathology, University of Miami School of Medicine, and Director of Clinical Pathology, Jackson Memorial Hospital, Miami, Florida. 1958, 735 pages, 6¾″ x 9¾″, 192 illustrations and 9 plates including 5 in color. Price, $13.75.

The C. V. MOSBY Company

3207 Washington Boulevard • St. Louis 3, Missouri

WHY Regent Lab Reagents Offer More!

- *ready for instant use*
- *packed dry—no water to pay for*
- *stable, sensitive, and rapid*
- *economical—low cost per test*

NESSLER COMPOUND

Simply dissolve in water
Already contains alkali
Solution stable indefinitely
For all methods of determining ammonia

NITROPRUSSIDE COMPOUND

Urine acetone test in seconds
One step—just add to urine
Sensitive—no false positives

BENZIDINE COMPOUND

Occult blood test in one minute
For blood detection in fluids and tissues
No glacial acetic acid used
Sensitive—no false positives

Laboratory Specialties Since 1946

Order from your dealer. Write for new catalog.

REGENT SCIENTIFIC CO., Inc.
P. O. Box 1036 Redondo Beach, California

SPECIAL DETERMINATIONS
FOR CLINICAL LABORATORIES

- HORMONE ASSAYS
- TOXICOLOGY
- ELECTROPHORESIS
- ENZYME AND LIPID CHEMISTRY
- PORPHYRINS—P B I

AIR MAIL SERVICE

Information, free schedules and mailing containers upon request

CLINICAL LABORATORY
OF LAS VEGAS
P.O. BOX 1227 • LAS VEGAS, NEVADA

CHANGING YOUR ADDRESS?

When you move, please—

(1) Notify us to change your address—allow us six weeks to make the change.
(2) Mention the name of this Journal. (We publish twelve periodicals.)
(3) Give us your old address. If possible, return the addressed portion of the envelope in which we sent your last copy.
(4) Give us your new address—complete—including the Postal zone number.
(5) Please print your name and address.

Thank You!

Circulation Department, The C. V. Mosby Company, Publishers, 3207 Washington Blvd., St. Louis 3, Mo.

You'll like this "Operation"!

Veeder-Root *Hand Tallies* for Quick Blood Count

Lab technicians like the operating speed and ease of checking red and white count and differential with Veeder-Root single and double counters. Nestled compactly and securely in either hand, other hand can adjust microscope, hold slide, dial phone or do pencil work. Slightest finger pressure registers each count. Just turn knob to zero for fast resetting.

QUICK FACTS — *Single* counter counts up to 10,000. White figures, black background.
Double Counter — Figures on one unit, white on black, other unit, red on white. Each unit counts up to 10,000. If not available from your Medical Supply House, use coupon below.

Veeder-Root
"The Name That Counts"
World's Largest Makers of Counting Devices

VEEDER-ROOT
HARTFORD 2, CONN.

Please send me descriptive folder of Veeder-Root Hand Tally Counters together with current prices.

Name.....
Street.....
City..... *State*.....

JL-8-59

HELP US KEEP THE THINGS WORTH KEEPING

You'd give him the world, if you could. A free and happy world to learn in. A world of peace, where he can grow up, free of fear.

But peace takes more than *wanting*, these days. It takes a lot of doing, too. And peace costs money.

Money for strength to keep the peace. Money for science and education to help make peace lasting. And money saved by individuals, to keep our economy sound.

You can do something about this. Every U.S. Savings Bond you buy helps provide money for America's Peace Power—to help us keep the things worth keeping.

Why not buy a few extra, in the months to come?

Photograph by Harold Halma

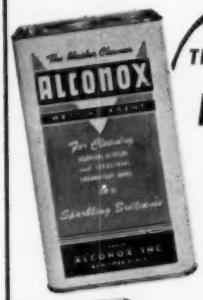
HELP STRENGTHEN AMERICA'S PEACE POWER

BUY U. S. SAVINGS BONDS

The U.S. Government does not pay for this advertising. The Treasury Department thanks The Advertising Council and this magazine for their patriotic donation.

What Is "ALCONOX-CLEAN?"

Just "clean" isn't good enough for glassware, instruments and equipment in the laboratory or hospital. Ordinary detergents may fail to do the job. But when it's "ALCONOX-CLEAN," you are sure!



SAVE TIME & MONEY with ALCONOX

the World's Most Thorough Cleaner!

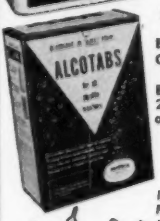
Proven Best by Test ★ For over 20 years
★ for wetting power
★ for sequestering power
★ for emulsifying effect
Yet it costs up to 75% less!

In 3 Convenient Forms

ALCONOX
For all equipment washed by hand
Box of 3 lbs. $1.95
Case of 12 boxes — 3 lbs. ea. ... $18.00

ALCOJET
For all equipment washed by machine
Box of 5 lbs. $3.00
Case of 6 boxes — 5 lbs. ea. ... $15.00

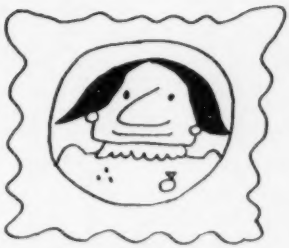
Both available in drums of 25, 50, 100 and 300 lbs. at additional savings!
(Prices slightly higher West of the Rockies)



ALCOTABS
In tablet form to clean PIPETTES in one easy operation. For all pipette washers.
Box of 100 tablets ... $5.00
Case of 6 boxes of 100 Tablets ... $30.00

Order from your Supplier or ask him for a sample!

ALCONOX, Inc.
853 Broadway, New York 3, N. Y.



Your great Aunt Amanda
as a lass was so chic

In her high button shoes
which to-day are antique.

Quite as passé
in a world that is new

Is a lab without PROTROL®—
Protrol's timing is true

Knickerbocker
300 West 43rd Street, New York City

Here's why PROTROL is perfect *every* prothrombin time . . .
Only Protrol is *human*
not a horse-plasma.
Only Protrol is *fresh*
not a synthetic-diagnostic.
Only Protrol is *pooled*
not a single variable plasma.
Use famous Protrol, prothrombin control reference standard, for greater accuracy in all your prothrombin time determinations.

COMPLEMENT FIXATION—Virus—Rickettsia—Fungus—RPCF

LIPID AND ENZYME CHEMISTRY
SPECIAL CHEMICAL PROCEDURES—P.B.I.—Mineral—Organic

72 to 96 hour service anywhere in the USA—Fee Schedules and Information on Request

Clinical Laboratory of San Bernardino

955 "D" Street, P. O. Box 329, San Bernardino, California

INDEX TO ADVERTISERS

Please mention "The Journal of Laboratory and Clinical Medicine" when writing to our advertisers—it identifies you

Albino Farms (Laboratory Animals) -- 31
Alconox, Inc. ---------- 39
Aloe Scientific ---------- 12
American Optical Company ---------- 17
American Red Cross ---------- 30
Ames Company, Inc. ---------- Fourth Cover

Biochemical Procedures ---------- 35

Chem-Tech Laboratories ---------- 35
Cherry Hill Farms (Laboratory Animals) ---------- 31
Clinical Laboratory of San Bernardino 40
Clinical Laboratory of Las Vegas ----- 37
Coleman Instruments, Inc. ---------- 8
Cook, Jay E., Importer (Xenopus Laevis Frogs) ---------- 33
Corning Glass Works ---------- 11

Difco Laboratories ---------- 1

Eaton Laboratories ---------- 2, 3

Fisher Scientific ---------- 10

Gradwohl School of Laboratory Technique ---------- 27

Hamilton Laboratory Animals, Inc. --- 33
Hamm Rabbitry, Paul (Rabbits and Guinea Pigs) ---------- 31
Hartman-Leddon Company ---------- 23
Hemlock Hollow Farm (Laboratory Animals) ---------- 31
Hyland Laboratories ---------- 5

Klett Manufacturing Co. ---------- 27
Knickerbocker Biosales, Inc. ---------- 39

Lab-Tek Plastics Co. ---------- 4
Lea & Febiger ---------- 18
Leitz, Inc., E. ---------- 21

Media, Inc. ---------- 20

New York Scientific Supply Co. (Laboratory Animals) ---------- 32
Nuclear-Chicago Corporation ---------- 7
Nutritional Biochemicals Corporation -- 27

Ortho Pharmaceutical Corporation ---- 13

Paragon C. & C. Co. Inc. ---------- 9, 15
Photovolt Corp. ---------- 29

Quivira Specialties Co. (Male Frogs)-- 32

Regent Scientific Co., Inc. ---------- 37
Research Supply Co. (Laboratory Animals) ---------- 32
Rolfsmeyer Co., Dan (Laboratory Animals) ---------- 33

Sanborn Company ---------- 19
Sargent & Co., E. H. ---------- 14
Schettle Frog Farm, Inc., J. R. (Laboratory Animals) ---------- 33
Scientific Products, Div. of American Hospital Supply Corp. ---------- 16
Standard Scientific Supply Corp. ------ 6
Steinhilber & Co., Inc., E. G. (Laboratory Animals) ---------- 32

Taconic Farms, Inc. (Swiss Mice) ---- 33
Technicon Company, The ----- Third Cover
Thomas Company, Arthur H. ---------- 24

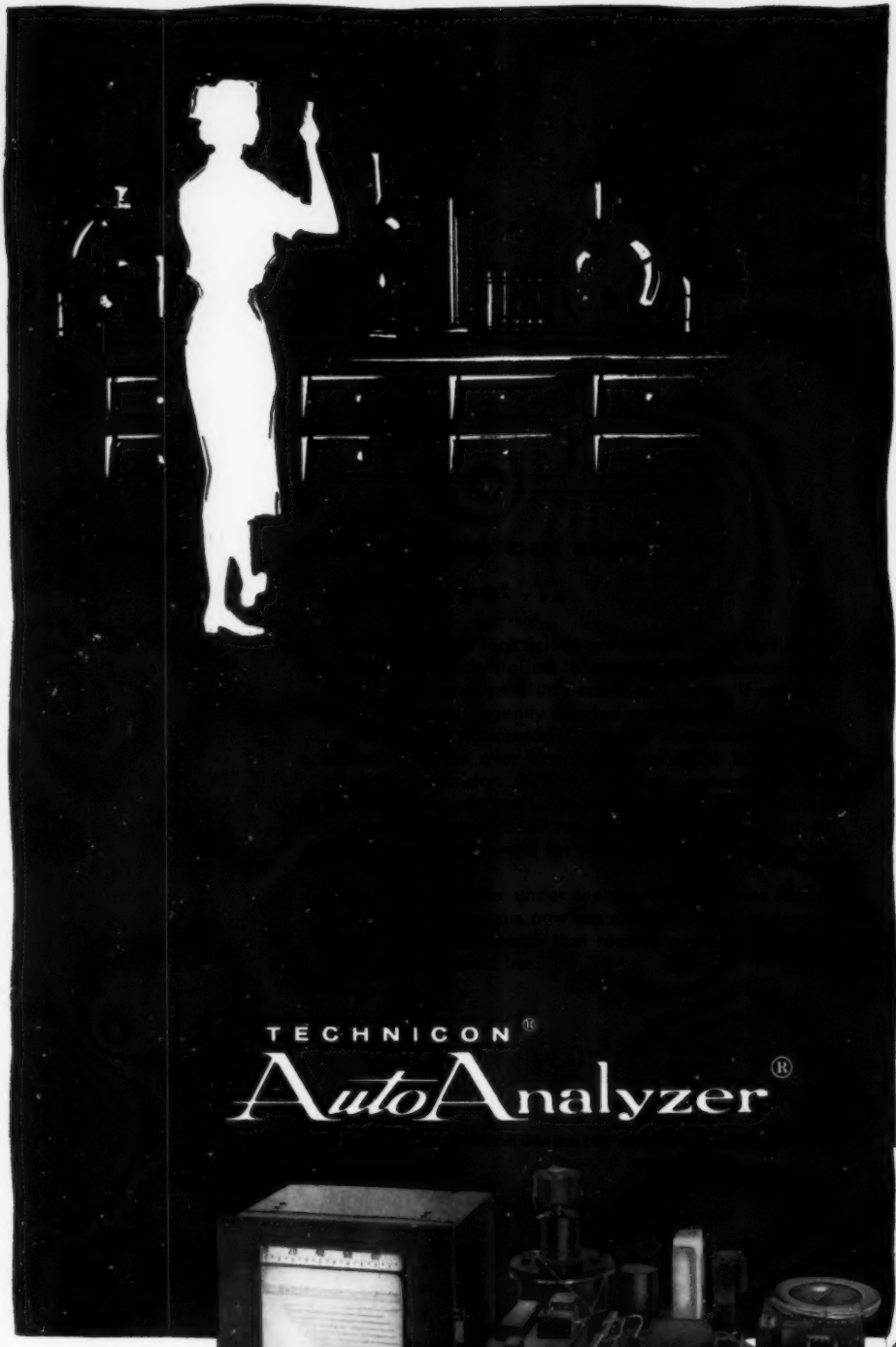
United States Savings Bonds ---------- 38

Veeder-Root ---------- 37

Warner-Chilcott Laboratories ---------- 25
Worthington Biochemical Corporation-- 29

Zeimet Bio-Farms ---------- 32

While every precaution is taken to insure accuracy, we cannot guarantee against the possibility of an occasional change or omission in the preparation of this index.



TECHNICON®

AutoAnalyzer®

Descriptive Booklet and Abstracts on AutoAnalyzer Methodology sent upon request . . . drop us a post-card.

Now

urinalysis made easier...

1 dip–3 test results

COMBISTIX®

Reagent Strips

colorimetric combination test for urinary protein, glucose and pH.

COMBISTIX *rapidly "screens" the urine* – just dip a single strip and read *protein* (in "plus" system or milligrams), *glucose* and *pH* answers within 10 seconds

- *colorimetric readings ELIMINATE GUESSWORK*– three standardized color charts provided
- *no more Q.N.S. reports*–only *drops* of urine required
- *no muss, no fuss, no cleanup* – completely disposable
- *no false positives* from turbidity interference, non-glucose reducing substances, drug metabolites

COMBISTIX utilizes a principle that enables more precise protein estimation, and an enzyme system specific for glucose. Selected indicators span the urine pH range, giving the reaction by pH *number* for more accurate reporting and as an aid in the identification of urinary crystals.

When work piles up and time runs out, it's good to know COMBISTIX can help speed the urinalyses reliably . . . and without water baths, burners, filtering, or test tubes to wash. You'll appreciate the *specificity* and *sensitivity* of COMBISTIX Reagent Strips as well as the speed.

Available: COMBISTIX Reagent Strips–Bottles of 125.

74059

AMES
COMPANY, INC
Elkhart • Indiana
Toronto • Canada